University of Alberta

Evaluation of Methods of Soil DNA Extraction for PCR

by

Valérie Chénard

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ABSTRACT

Different methods of direct DNA extraction and purification from soil samples were investigated on four soil types. The method using Sephacryl S-400 HR MicroSpin™ columns was the most robust to yield DNA suitable for PCR amplification using the fungal universal primers ITS1-F/ITS4. Some physical and chemical soil characteristics were determined and related to DNA yield and quality, and PCR amplification success. The A_{260}/A_{230} and the A_{260}/A_{280} ratios, and the PCR success, were significantly affected by the method of DNA extraction used.

The developed method was used to extract DNA from soil from five *Populus tremuloides* Michx. stands in an attempt to study the diversity of the endomycorrhizal fungi community. A nested PCR was performed using primers NS1/NS8 first, followed by primers VANS1/NS4. However, the first PCR usually gave weak or no amplification so it is suggested to determine the sensitivity of the method and improve PCR conditions for enhanced specificity.

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LIST OF ABBREVIATIONS

A_{230}:	Absorbency at 230 nm
A_{260}:	Absorbency at 260 nm
A_{280}:	Absorbency at 280 nm
ALPAC:	Alberta-Pacific Forest Industrie Inc.
AM:	arbuscular mycorrhizal or arbuscular mycorrhizae
BGL:	Brunisolic Grey Luvisol
CsCl:	caesium chloride
CTAB:	hexadecyltrimethylammonium bromide
ddH_2O:	double distilled water
DF:	degree of freedom
dH_2O:	distilled water
DNA:	deoxyribonucleic acid
EDTA:	ethylenediaminetetraacetate
EEB:	Eluviated Eutric Brunisol
EMEND:	Ecosystem Management by Emulating Natural Disturbance
EtBr:	ethidium bromide
FA:	fulvic acids
FA-A:	conventional fulvic acids
FA-B:	clay-associated fulvic acids
GEBC:	Gleyed Eluviated Black Chernozem
GPS:	Geographic Positioning System
HA:	humic acids
HA-A:	conventional humic acids
HA-B:	clay-associated humic acids
IPTG:	isopropyl β-thiogalactopyranoside
ITS:	internal transcribed spacer
LB:	Luria Bertani
ND:	not determined
N.E.:	North East
PCR:	polymerase chain reaction
PEG:	polyethylene glycol
PVP:	polyvinylpyrrolidone
PVPP:	polyvinylpolypyrrolidone
R.:	range
rDNA:	ribosomal DNA
rev:	revolutions
RLFP:	restriction fragment length polymorphisms
rRNA:	ribosomal RNA
SDS:	sodium dodecyl sulfate
sec.:	section
SS:	sum of squares
SSU:	small subunit rDNA
TAE:	Tris-acetate EDTA
TBE:	Trid-borate EDTA

TF:	Typic Fibrisol
Tp.:	township
vol:	volume
W.4:	West 4th Meridian
W.5:	West 4th Meridian
wt:	weight
x-gal:	5-bromo-4-chloro-3-indolyl-β-D-galactoside
%humin:	percentage of humine
%oc:	percentage of organic carbon
%om:	percentage of organic matter
%tn:	percentage of total nitrogen
18S:	small subunit rDNA

CHAPTER 1

GENERAL INTRODUCTION

1.1 Introduction

Microorganisms drive the chemistry of natural environments, and without them life would not be possible on the planet (Ritz *et al.*, 1994). Environmental monitoring of microorganisms is necessary to follow the activities of microorganisms with genes encoding specific metabolic activities and also for public and environmental health. Among those microorganisms, mycorrhizal fungi are deemed essential in the establishment of plant species and act as keystones that contribute to the ecological resilience, evolutionary potential, and consequently, to the sustainability of forest ecosystems (Smith and Read, 1997). Therefore, the ability to monitor individual species or complex communities is important, because factors such as increased levels of pollution and global warming effects may upset the balance of communities and possibly their functions (Rygiewicz and Andersen, 1994).

Until recently, studies of soil ecology and microbiology relied on conventional optical microscopic observations and cultivation-based approaches. For example, these methods were used to study the microbial diversity or to screen a group of organisms possessing the ability to metabolize certain compounds. The most commonly cited limitation of these approaches stems from the finding that the majority of microbial cells cannot be cultured on artificial media and therefore, gives only a partial view of the total diversity of microorganisms or of a particular group of microorganisms present in a particular environment. For example, only a small fraction of bacteria (sometimes 0.5% or less) have been cultivated in the laboratory, as determined by plate count (Torsvik *et al.*, 1990; Cullen and Hirsch, 1998), compared to the total bacteria count, determined by fluorescent microscopy after staining with acridine orange (Torsvik *et al.*, 1990).

The advent of molecular techniques has revolutionized our understanding of microbial ecology. Indeed, newer methods based on the use of molecular biology techniques to analyse total extracted DNA (deoxyribonucleic acid) from natural environments (e.g., soil samples) potentially sample the entire population, and thus, may provide a more representative picture of the total microbial community. Some molecular approaches examine cellular components such as phospholipid fatty acids, whereas others are directed at analysing nucleic acids either *in situ* or following nucleic acid extraction (Bruce *et al.*, 1999). There is no doubt that application of new DNA methods for soil analyses heralds a rich future for recognizing new mycorrhizal species (or any other organisms), understanding how they interact in complex communities, and how their functions can be understood and possibly manipulated for environmental biotechnological purposes.

DNA extraction from soil samples (or any other samples) is one of the ways to study microbial communities. When direct DNA extraction is used, the DNA extract contains the DNA from most of the organisms present in the sample, except the DNA from some organisms that are recalcitrant to cell lysis, such as small coccoid bacterial cells (Moré *et al.*, 1994). When a specific organism or group of organisms is targeted and if a pair of primers specific for that organism or group of organisms is available, its DNA can be amplified using the polymerase chain reaction (PCR).

The development of PCR in 1983 (Mullis *et al.*, 1986; Mullis and Faloona, 1987; Mullis, 1990 - as cited by Atlas *et al.*, 1992) is an example of a major methodological breakthrough in molecular biology. This relatively new technique permits the *in vitro* replication of specific sequences of DNA whereby gene segments can be amplified. The exponential DNA amplification nature of this method enhances gene probe detection of defined rare gene sequences in heterologous mixtures of DNA. Depending on the targeted DNA and primer nucleotide composition, the amount of each component needs to be optimized to decrease the formation of primer-dimers and non-specific PCR products. The formation of primer-dimers and non-specific PCR products reduces the

yield of the desired product by using primers and nucleotides that would otherwise be used for the synthesis of the specific PCR product.

Once amplified, the DNA can be identified on the basis of the PCR product(s) length, the sequence of the PCR product(s), or DNA hybridization. As previously mentioned, this technique can be very useful to identify organisms that are not cultivable or that are not easily identified by morphological characteristics, such as in the case of mycorrhizal fungi. The identification of mycorrhizal fungi has been mostly based on morphological differences, which can be of limited efficiency because two different species or genera might have similar characteristics. Another limitation is that all their vegetative and reproductive structures are not always present at the time of sampling. Thus, a method of identification based on direct DNA extraction from soil would overcome some of the limitations encountered by other more conventional methods.

Several methods of DNA extraction and purification from soil samples were developed in the last few years. Some methods require enormous amounts of material (e.g., Steffan *et al.*, 1988), others are very time consuming (e.g., Steffan *et al.*, 1988; Tsai and Olson, 1991; Young *et al.*, 1993), and some use chemicals that can be harmful to humans and the environment (e.g., Steffan *et al.*, 1988; Tsai and Olson, 1991). Also, some methods do not work very well with soils with high amounts of organic matter (Zhou *et al.*, 1996). That is why the development of faster, more efficient and safer methods of DNA extraction from soil samples is important, not only for molecular identification of mycorrhizal fungi, but the identification of other organisms as well.

1.1.1 General characteristics of Populus tremuloides

The genus *Populus* is one of two members of the family Salicaceae and is comprised of six taxonomically distinct sections, consisting of 29 species with a wide transcontinental distribution in the Northern Hemisphere (Stettler *et al.*, 1996; Dickmann, 2001a). All taxa in the genus are dioecious, with male (pollen-bearing) and female (seed-producing) flowers, both of which are borne on pendant catkins, typically occurring on separate trees

but there are occasional hermaphrodite clones bearing bisexual catkins (Dickmann, 2001a).

Poplars are deciduous or (rarely) semi-evergreen forest trees and are short-lived compared to other trees such as white pine, oaks (*Quercus*), or Douglas-fir (*Pseudotsuga menziesii* [Mirb.] Franco) (Dickmann, 2001a). Because many poplars have a large natural range, they often segregate into geographically and morphologically distinct subspecies and varieties (Dickmann, 2001a). Twelve species are indigenous to North America (Dickmann, 2001a). *Populus tremuloides* Michaux (or Michx.), which is often called quaking aspen or trembling aspen (Peterson and Peterson, 1992), is part of the section *Populus* (formerly Leuce section) (Dickmann, 2001a), subsection Trepidae (Dickmann and Stuart, 1983; Peterson and Peterson, 1992) and comprises five varieties: *vancouveriana* (Trel) Sarg. (Vancouver Island form), *aurea* (Tidestr.) Daniels (Rocky Mountains form), *pendula* Jaeger & Beissner, *magnifica* Vict., and *reniformis* Tidestr (Eastern forms) (Dickmann, 2001a).

Trembling aspen is a slender tree with straight to twisted form and a small rounded crown (Dickmann, 2001a), has a relatively gradual taper in its stems (Peterson and Peterson, 1992), and often reaches 20 m or more (Dickmann and Stuart, 1983). It has a distinctive greenish-white to creamy, yellowish-gray, yellowish-brown, gray, or green bark, which is frequently darkened by warty bands (Peterson and Peterson, 1992; Dickmann, 2001a). The green hue evident in many clones is caused by the presence of chlorophyll in the bark (Peterson and Peterson, 1992).

Leaf morphology is the diagnostic feature that is most useful for distinguishing one *Populus* species from another in the summer in Canada (Peterson and Peterson, 1992). In autumn, coloration of poplar leaves generally is yellow or pale gold, although in the West, some clones of *P. tremuloides* turn bright orange (Dickmann, 2001a). Identification can also be done on twigs and buds in winter (Peterson and Peterson, 1992). At maturity, quaking aspen have small, round to oval leaves that have finely toothed margins, but young suckers have much larger and more elongated leaves

(Peterson and Peterson, 1992). The property of the leaves to flutter easily is caused by the flatness of the petiole (Dickmann and Stuart, 1983), which acts as a pivot for the blade (Peterson and Peterson, 1992).

The morphology of aspen shoots, buds, leaves, aments, and flowers has been summarized by Peterson and Peterson (1992). Leaves on long shoots contribute the most to growth of a poplar tree because of their long duration, abundance, large area, and high photosynthetic rate (Dickmann *et al.*, 2001). In general, aspen in closed stands have shallow crown systems and relatively low branch biomass (Peterson and Peterson, 1992). The rapid growth of young aspen stands is in part due to the high net difference between photosynthesis and respiration rates, which is due to the large leaf system relative to the amount of respiring tissue present in the stem, branches, and leaves (Peterson and Peterson, 1992).

In even-aged aspen stands, there often is a wide range of diameters because of variations in site quality and competition by surrounding trees, which make it difficult to correlate tree age and diameter (Hiratsuka and Loman, 1984). Also, growth in clonal trees may occur through addition of new suckers and annual ring width growth (Peterson and Peterson, 1992), and an increasing number of stems in a clone has been associated with decreasing ring growth in male aspen clones (Sakai and Burris, 1985).

Aspen is a highly shade and competition intolerant species and is a common pioneer on sites disturbed by logging, fire, or other natural disruptions (Dickmann, 2001a), as well as after mining activity (Cripps, 1997). Aspen is often considered as a nurse crop for conifers in the boreal mixedwoods ecosystem because of the shade it provides for understory species and other tree species that do not easily establish in full sunlight (Peterson and Peterson, 1992). As a pioneer, trembling aspen can form extensive pure, even-aged stands, but two-storied or uneven-aged stands also occur in the Rocky Mountains, and mixed even-aged with other hardwoods and conifers commonly occur, especially in the more southerly parts of its range (Dickmann, 2001a).

Poplars typically have two sets (diploid) of 19 chromosomes (2n = 38) (Dickmann, 2001a). However, *P. tremuloides*, along with *P. tremula* and *P. alba*, can occur naturally in a triploid form (3n=57) (Dickmann and Stuart., 1983), and even in a tetraploid form (Every and Wiens, 1971).

1.1.2 Reproduction of Populus tremuloides

Although aspen do regenerate from seeds, stands of clonal origin predominate (Peterson and Peterson, 1992). The ability to reproduce both sexually (seedling) and asexually (sucker) gives aspen an advantage over boreal conifers. Seedlings and suckers occur at different places, different times, or under different circumstances, and Peterson and Peterson (1992) hypothesized that many aspen stands are probably a result of concurrent development of seedling- and sucker-origin stems. It is possible to distinguish aspen of seedling and sucker origin by the difference in root morphology (Peterson and Peterson, 1992).

In some naturally growing poplars, the original ancestor, which is the original seed-derived donor plant of a clone, may have originated millennia ago and have persisted through thousands of self-replications, generating offspring (Dickmann, 2001a). The clonal offspring have the characteristic to be the exact genetic copies of their parents, provided that no mutation occurs to any individual (Dickmann, 2001a). The vegetative propagation nature of poplars and other plant species enables many to successfully compete and reproduce in their ecological habitat and also can be an effective strategy for invading new habitats (Dickmann, 2001a). There are clone-to-clone variations in many features of aspen (summerized by Peterson and Peterson, 1992).

The ideal time to identify clones is during the period of colour change and leaf fall in early autumn and the period of leafing out in late spring (Peterson and Peterson, 1992). Genetic studies involving electrophoretic analysis of isoenzymes are very precise techniques for clonal differentiation (Cheliak, 1980 - as described by Peterson and Peterson, 1992; Cheliak and Dancik, 1982; Cheliak and Pitel, 1983). The ability to

differentiate different aspen clones is important for management, because superior clones can be several times more productive and more resistant to diseases than inferior ones (Peterson and Peterson, 1992).

Most poplars will produce large amount of sprouts from the root collar or a cut stump of a tree that has been killed, but this ability decreases as the tree matures (Dickmann, 2001a). Living trees also will send up suckers from roots that have invaded adjacent open areas (Dickmann, 2001a). Root suckers are the main way that poplars vegetatively reproduce and in aspens, these shoots are produced copiously from the shallow, widespreading, horizontal root system after the stand is logged, killed by fire, or windthrown (Dickmann, 2001a). After clearcutting, from 25 000 to 75 000 suckers per hectare can develop within 2 years (Newcombe *et al.*, 2001). However, it is not uncommon in the Prairie Provinces to observe a reduction in suckers of 80% from year 1 to year 5 (Peterson and Peterson, 1992). Suckering produces clones that can vary in size, from several trees to many thousand, sometimes producing a forest of clonal offspring (Dickmann, 2001a). Even in the absence of aspen canopy, aspen roots may persist, nurtured only by transient suckers beneath the coniferous canopy (Schier *et al.*, 1985). Several factors control aspen suckering (summerized by Peterson and Peterson, 1992).

The suckers originating from a root system of a parent tree remain connected by parent roots, at least for the first few decades, even after they have developed their own root system, until one of the trees dies (Peterson and Peterson, 1992), although some connections will likely decay and break (Barnes, 1959; Gifford, 1966 - as described by Peterson and Peterson, 1992). New suckers depend upon the parent root for nutrients and water for at least 25 years following establishment (Zahner and DeByle, 1965), which give them a growth and survival advantage over seedlings of aspen and other species (Graham *et al.*, 1963). There is a negative relationship between the formation of new roots in regenerating stands and the mass of the parent root system (DesRochers, 2000). This suggests that if arising suckers do not have to spend energy to produce new root systems, they are likely to invest more energy into height and leaf area growth.

Usually, aspen suckers arise from horizontal lateral roots that have a diameter between 0.5 and 2 cm or on points along a lateral root where it has tapered to less than 2 cm (Peterson and Peterson, 1992). This horizontal root becomes enlarged on the distal side that faces away from the parent tree (Dickmann *et al.*, 2001). Young roots, therefore, can produce a large number of suckers, which suggests that suckers would be more likely to be concentrated near the outer parts of parental root systems. Generally, most of the suckers are produced during the first growing season after a major disturbance such as fire or harvesting (Peterson and Peterson, 1992). Their growth is very rapid during the first few years (Dickmann *et al.*, 2001).

New roots are rapidly formed as the root-absorbing area quickly expands during the juvenile stage of growth, but formation declines and reaches somewhat of an equilibrium as trees mature (Dickmann *et al.*, 2001). Death most frequently occurs in the youngest roots, probably because they are most susceptible to pathogenic fungi and grazing by soil arthropods and other root herbivores (Dickmann *et al.*, 2001).

Trembling aspen generally reaches maturity after 30 to 40 years. An old tree can produce over 50 million seeds in a single season (Dickmann, 2001a). Aspen can flower as young as 10 years, but 15 years is more common (Maini, 1968 - as described by Peterson and Peterson, 1992). Catkins emerge before the flush of leaves in the spring, and flowers appear at the very beginning of spring (Dickmann, 2001a). After wind pollination, the infructescence quickly matures, and when it is ripe, capsules split into two, three, or four parts, and the seeds take to the air in May and June and can be carried several kilometres by the wind (Dickmann and Stuart, 1983; Peterson and Peterson, 1992). The seeds of aspens are light and extremely small, averaging 6.6 million seeds kg^{-1} (Dickmann and Stuart, 1983). Each seed is surrounded by tufts of long, white, silky hairs attached to the basal end.

The germinative capacity of aspen seeds at seedfall usually exceeds 95% (Schopmeyer, 1974), although their viability is variable and may decline after several weeks (Dickmann and Stuart, 1983). The requirements for seed establishment are stringent. The seeds need

to be in immediate contact with moist soil to absorb water and nutrients (Borset, 1960 - as described by Peterson and Peterson, 1992). The seedbed should be slightly acidic and be kept moist at all times (Dickmann and Stuart, 1983). Lack of competition and low evapotranspiration also are important factors for successful establishment (Peterson and Peterson, 1992). Seedlings grow relatively slowly for the first 2 or 3 years (Heeney *et al.*, 1975) and their early height growth is slower compared to suckers (Peterson and Peterson, 1992).

1.1.3 Distribution of Populus tremuloides

Trembling aspen is found in all provinces of Canada (Peterson and Peterson, 1992) and is the most widely distributed tree species in North America, growing transcontinentally through Canada to Alaska and in the northern states, the Rocky Mountains, and the Cascades Mountains in the United States (Dickmann, 2001a) (Fig. 3.1). The wide distribution of quaking aspen explains the variation within this species, which may be the most genetically variable plant species ever studied (Mitton and Grant, 1996).

It is in the Rocky Mountains that we can find what some feel are the most impressive stands of *P. tremuloides*, where trees can attain heights of 30 m or more and a diameter of nearly 1 m, whereas at high elevations, high latitudes, on south- and west-facing slopes, or on the prairie fringe, trees may only be a twisted shrub (Dickmann, 2001a). The wide distribution of trembling aspen has been hypothesised by Cripps and Miller (1993) to be due, at least in part, to its association with a very wide variety of ectomycorrhizal fungi.

Fig. 1.1. Natural distribution of aspen in North America (from Peterson and Peterson, 1992 - adapted from Little, 1971).

1.1.4 Site and condition requirements of Populus tremuloides

Aspens will grow almost everywhere, from wet clayey soils to coarse, droughty sands, but because they are highly exploitative of water and nutrients, they will reach their full

potential only on well-drained, loamy soils high in lime with a water table within 1.5 m of the surface (Dickmann *et al.*, 2001). Aspen trees are considered an upland species adapted to sandy soils, but will frequently be found growing in swamps and bogs. However, on the latter sites vigour and growth rate will probably not be impressive compared to that on a good quality site (Dickmann and Stuart, 1983). Aspen plays a major ecological role in the colonization of upland areas burned by intense, stand-replacing fires, where its seeds germinate readily on the ash-covered soil.

The biological productivity of poplar clones is mostly determined by climate, which includes solar radiation, temperature, rainfall, humidity, and wind. Warm and sunny areas are more productive than cool and cloudy ones, and on non-irrigated sites, water is the main factor that limits productivity (Dickmann *et al.*, 2001). When the ratio between the amount of precipitation and the rate of evaporation of water (P/E ratio) falls below 1 during the growing season, the productivity of the stand will suffer (Dickmann *et al.*, 2001). The relation between climate and the periodic biological activity of plants, referred to as phenology, also determines the yield (Dickmann *et al.*, 2001).

The ideal site for aspen growth will have an optimum pH that ranges from 5.5 to 7.5, and will be deep and medium-textured (sand/loam) to let the roots penetrate to at least 1 m without being interrupted by bedrock, water table, hardpan, or a gravel layer (Dickmann and Stuart, 1983; Stanturf *et al.*, 2001). However, genetic variation also has a major role in determining if a particular clone will grow well or not (Dickmann and Stuart, 1983).

1.1.5 Properties and uses of Populus tremuloides *wood and forests*

The wood of trembling aspen is light in colour and weight (except for a dark-coloured heartwood or wetwood core), soft, straight-grained and uniform in texture, and has low-density, with an average specific gravity of about 0.35 (Balatinecz and Kretschmann, 2001; Dickmann, 2001a). It has low lignin content and high carbohydrate content (Micko, 1987, as cited by Peterson and Peterson, 1992).

Populus species are among the fastest-growing trees in the temperate latitudes (Dickmann and Stuart, 1983; Eckenwalder, 1996). More specifically, aspen and balsam poplar (*Populus balsamifera* L.) are major components of the forest resources in the Prairie Provinces and in northeastern British Columbia in Canada (Peterson and Peterson, 1992). Because of its abundance and wide distribution, the wood of aspen is one of the most important raw wood materials in Canada and the Lake States (Dickmann, 2001a). Aspen wood is mostly used for pulp, paper, hardboard, dimension lumber, and insulation board as well as waferboard and oriented strandboard, and other wood products (Dickmann, 2001a; Balatinecz and Kretschmann, 2001).

Aspen also have a very important role in giving some protection against rapid wildfire advance (Amacher *et al.*, 2000), in reducing erosion on upland and floodplain sites, in providing habitat for wildlife and fish, and being aesthetically attractive (Amacher *et al.*, 2000; Dickmann *et al.*, 2001). In addition to feeding ungulates, aspen also provides food and prime habitat for ruffed grouse and beaver, and hosts other animals (e.g., squirrels) and birds (Dickmann, 2001a). In the Rocky Mountains, indigenous peoples were also using trembling aspen for a variety of uses (Dickmann, 2001a).

1.1.6 Causes of decline of Populus tremuloides *stands*

The area of disturbance-dependent quacking aspen is almost everywhere on the decline because of fire exclusion, severe browsing by deer and elk, land clearing for agriculture, and the inability to log stands (which reach decadent stage, die, and then succeed to another vegetation type) (Bartos and Campbell, 1998; Dickmann, 2001b). However, aspens are in no danger of disappearing and their population is even increasing in the boreal mixedwood region in Canada (Dickmann, 2001b). *Populus. tremuloides* sensitivity to the levels of CO_2 and O_3 ranges from very sensitive to very tolerant, but on the whole, expected air pollution and elevated CO_2 will result in a decrease of aspen growth (Isebrands and Karnosky, 2001).

1.1.7 Enemies of Populus tremuloides

Trembling and largetooth aspens are hosts to a large number of insect species including defoliators and sucking insects, as well as wood and bark borers (Thomas and Rose, 1979), some causing highly conspicuous blights and in some years, premature defoliation (Newcombe *et al.*, 2001). In North America, there are at least 300 species of insects and mites commonly found on the 12 species of *Populus* (Peterson and Peterson, 1992). Moreover, the number of pathogens on *P. tremuloides* alone is just as high, with more than 250 species of fungi causing decay (Newcombe, 1996). Among the insects, only a handful is seriously threatening.

Cervid species use poplars as a preferred browse and can cause establishment failure, especially where browsing pressure is high (Stanturf *et al.*, 2001). The barking of aspen can provide a point of entry for canker fungi and other pathogens and insects. High vole (*Microtus* spp.) populations, which feed on roots and lower stems, also can lead to heavy mortality (Stanturf *et al.*, 2001). Aspen accumulate large amounts of phenolic glycosides (salicylates) in most tissues, and coniferyl benzoate in flower buds, which act as chemical defences against environmental damaging agents (Lindroth, 2000).

1.1.8 Mycorrhizal fungi colonization of Populus tremuloides

Trembling aspen (*Populus tremuloides* Michx.) is colonized by both endomycorrhizal and ectomycorrhizal fungi (Khasa *et al.*, 2002). This symbiotic association is beneficial in several ways to aspen by augmenting inorganic nutrient uptake and providing protection from heavy metals, drought, pathogens, grazers, and other organisms (Fogel, 1980) therefore, assessing the diversity of mycorrhizal fungi in aspen stands that perform well could have potential benefits for sustainable management of this tree species, as well as general knowledge of the dynamics of its ecosystems.

1.2 Research objectives

This study had two objectives:

1) The first goal was to investigate different methods of direct DNA extraction and purification in an attempt to develop an environmentally friendly (e.g., using as few and as safe reagents as possible), simple, rapid and reliable method for obtaining high-molecular-weight DNA from different soil orders for PCR amplification.

2) The second goal was to use the developed soil DNA extraction method to study the endomycorrhizal fungal diversity of trembling aspen forests in northern Alberta and Québec. For this, the purified DNA had to be amplified using the PCR technique, and the resulting PCR fragments had to be cloned and then sequenced.

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CHAPTER 2

EVALUATION OF DIFFERENT METHODS OF DIRECT SOIL DNA EXTRACTION FOR PCR AMPLIFICATION APPLICATIONS

2.1 Introduction

Two general types of methods are used to extract DNA from environmental samples: direct methods and indirect methods (Steffan *et al.*, 1988). Indirect methods separate cells of interest prior to cell lysis (e.g., by sucrose gradient centrifugation), whereas direct cell lysis methods occur within the environmental matrix, providing an extracted DNA from live and dead cells (eukaryotic and prokaryotic cells), in addition to any extractable extracellular DNA persisting in the sample (Bruce *et al.*, 1999). Direct lysis, first described by Ogram *et al.* (1987), is used more often because it gives a higher yield of DNA (Steffan *et al.*, 1988; Atlas *et al.*, 1992; Cullen and Hirsch, 1998), more samples can be processed at the same time, and it avoids biases of breaking fragile cells that can happen during cell extraction (Cullen and Hirsch, 1998). It is also assumed to produce molecules that are representative of microbial communities. Several methods of DNA extraction and purification from soil samples were developed in the last few years. Some methods are very time consuming (e.g., Steffan *et al.*, 1988; Tsai and Olson, 1991; Young *et al.*, 1993), others require an enormous amount of material (e.g., Steffan *et al.*, 1988), and some use chemicals that can be harmful to humans and the environment (e.g., Steffan *et al.*, 1988; Tsai and Olson, 1991), such as the use of ethidium bromide (EtBr) or phenol. Also, some methods do not work very well with soils with a high amount of organic matter (Zhou *et al.*, 1996).

Lysis of cells can be achieved by several methods. These include chemical treatments such as lysozyme, proteinase K (Torsvik *et al.*, 1990; Cullen and Hirsch, 1998), and sodium dodecyl sulphate (SDS)-lysis buffer (NaCl, Na_2EDTA, SDS) (Porteus *et al.*, 1997), and physical treatments such as Mini-Beadbeater (Berthelet *et al.*, 1996), hot-SDS

lysis, freeze-thaw lysis (Cullen and Hirsch, 1998), or a combination of the above methods. The rapid bead-beating, however, may shear DNA and this may result in small fragments that cannot be used for the PCR because they may form chimeric hybrids during amplification (Porteus *et al.*, 1997). To prevent shearing of DNA, the minimum time of beating required to lyse the cells should be used. High yield of high molecular-weight DNA can be a challenge with soils high in clay or organic matter because microbial cells may remain tightly bound to soil colloids. Thus, differences in soil characteristics, as well as bacterial community composition, are causes of variation in cell lysis efficiency (Zhou *et al.*, 1996). Criteria that can be used to determine lysis efficiency include the loss of organism viability, the total DNA yield, the total count of organisms (by microscopic examination), and morphological diversity changes (because some organisms are lysed more easily than others) (Moré *et al.*, 1994).

Another critical step in DNA extraction from soil is purification, because many contaminants can inhibit *Taq* polymerase during the polymerase chain reaction (PCR) (Berthelet *et al.*, 1996), interfere with restriction endonuclease digestion (Steffan *et al.*, 1988) and DNA hybridization specificity (Cullen and Hirsch, 1998), and reduce transformation efficiency (Tebbe and Vahjen, 1993). The presence of environmental contaminants may significantly affect detection by PCR when only a low copy number of target DNA is present in a sample (Jacobsen, 1995). When DNA is extracted by the direct method, it is highly contaminated with humic substances that covalently bind to it (Porteus *et al.*, 1997), which can interfere with DNA detection and measurement (Zhou *et al.*, 1996). Several techniques to purify DNA have been tested. These include ion-exchange chromatography on DEAE-Sepharose CL-6B (Torsvik, 1980), hydroxyapatite column chromatography (Torsvik, 1980; Steffan *et al.*, 1988), cetylpyridinium bromide precipitation (Torsvik *et al.*, 1990), caesium chloride-ethidium bromide (CsCl-EtBr) density gradient centrifugation (Steffan *et al.*, 1988; Bruce *et al.*, 1999), glassmilk and spearmine-HCl (Smalla *et al.*, 1993), and polyethylene glycol (PEG) 8000 precipitation (Steffan *et al.*, 1988; Porteus *et al.*, 1997). Other techniques have also been used such as theMicrocon-100^{TM} microconcentrator (Amicon, Beverly, MA, USA) (Widmer *et al.*, 1996; Porteus *et al.*, 1997), phenol-chloroform-isoamyl alcohol (Steffan *et al.*, 1988; Tsai

and Olson, 1991), (lengthy) cold isopropanol precipitation and Elutip-d^{TM} columns (Tsai and Olson, 1991), Sephadex G-200 columns (Tsai and Olson, 1992b), Sephadex G-100 and Chelex 100 resins (Moré *et al.*, 1994), addition of potassium acetate (Steffan *et al.*; 1988, Claassen *et al.*, 1996; Porteus *et al.*, 1997), addition of polyvinylpolypyrrolidone (PVPP) in extraction buffer (Claassen *et al.*, 1996), lysed cell extracts (Steffan *et al.*, 1988) or spin-columns (Berthelet *et al.*, 1996; Cullen and Hirsch, 1998), and labour-intensive agarose gel purification (Zhou *et al.*, 1996), which might include polyvinylpyrrolidone (PVP) (Young *et al.*, 1993).

Some of these procedures (e.g., hydroxyapatite column and CsCl density gradient centrifugation) decrease DNA yield, are labour-intensive (e.g., agarose gel purification) or toxic to humans (e.g., phenol, EtBr). For example, the DNA extraction procedure used by Volossiouk *et al.* (1995), which used skim milk powder and liquid nitrogen grinding, yielded DNA extract that could be used directly in PCR without additional need for purification. However, this method included the use of a solution of phenol.

The addition of water-soluble PVP in agarose gel retards the electrophoretic mobility of denaturing phenolic groups, such as the ones present in humic acids, so that they do not co-migrate with DNA (Young *et al.*, 1993). The addition of water-insoluble PVPP to soil buffer slurry prior to cell lysis (Atlas *et al.*, 1992), as well as the PVPP spin columns, also yields pure preparations of DNA from soil (Berthelet *et al.*, 1996). PVPP forms hydrogen bonds with phenolic compounds present in the structure of humic substances, which generates a PVPP-phenolic precipitate that can be removed by centrifugation (Young *et al.*, 1993). Steffan *et al.* (1988) showed that humic acids mask the detection of DNA and that PVPP reduces (but does not completely remove) the amount of humic compounds. Their results also suggested that the inclusion of PVPP treatment appeared to be both warranted and a necessary modification to the procedure originally described by Ogram *et al.* (1987). However, the results obtained by Zhou *et al.* (1996) have shown that hexadecyltrimethylammonium bromide (CTAB) also complexes with, and removes humic substances from DNA but unlike PVPP, it does not result in DNA loss. Humic acids have a core of strongly condensed aromatic structures surrounded by aliphatic side-

chain components and are believed to be the most biologically resistant fraction of organic matter (Anderson and Schoenau, 1993). Some humic acids have molecular weights of 100 000 or greater (Zhou *et al.*, 1996). The phenolic groups of humic acids denature biological molecules by bonding to N-substituted amides or oxidize to form a quinone that covalently bonds to DNA or proteins (Tsai and Olson, 1992a). Pure humic acids can inhibit PCR at a concentration as low as 10 ng (Tsai and Olson, 1992b). It is possible that humic acids originating from different soils may have different inhibitory properties (Jacobsen, 1995).

Moré *et al.* (1994) showed that Sephadex G-100 and Chelex 100 resins successfully remove PCR-inhibitory substances from groundwater concentrates. Porteus *et al.* (1997) reported that PEG 8000 is preferable to isopropanol early in the extraction because alcohol facilitates co-purification of DNA with humic material. However, Cullen and Hirsch (1998) recommend isopropanol to concentrate soil DNA from soil samples because there are no additional steps required for its removal after DNA precipitation. Potassium acetate can be useful to enhance the precipitation of DNA from soil proteins and lipids, and CTAB and chloroform remove plant and fungal polysaccharides and denature proteins from DNA (Porteus *et al.*, 1997).

After these extraction and purification procedures, the yield (concentration) and purity of DNA may be determined. Cullen and Hirsch (1998) measured the fluorescent emission of DNA with an MSE Spectroplus-D spectrophotometer and the absorbency of the DNA preparation at 230 nm (A_{230}), 260 nm (A_{260}), and 280 nm (A_{280}). A A_{260}/A_{280} ratio higher than 1.7 and a A_{260}/A_{230} ratio higher than 2.0 indicate a relatively pure DNA preparation. On the contrary, lower A_{260}/A_{230} ratios indicate the presence of humic substance contamination and lower A_{260}/A_{280} ratios indicate the presence of protein contamination. To be accurate, sample dilution should be adjusted with distilled water (dH_2O) or an appropriate buffer so that absorbency readings fall between 0.1 and 1.0. An absorbency of 1.0 at A_{260} corresponds to 50 $\mu g\ ml^{-1}$ of double-stranded DNA. Moreover, Cullen and Hirsch (1998) ran aliquots of this DNA in an agarose gel that was further stained in EtBr

(0.4 mg l^{-1} dH_2O), de-stained in dH_2O and photographed with Polaroid type 665 under UV illumination (312 nm) to estimate the quantity and size range of DNA fragments.

When the DNA is pure enough, various procedures can be carried out such as restriction endonuclease digestion analysis, hybridization analysis and dot blot analysis. The level of purity needed depends on the analysis done and the conditions used. More recently, PCR has become a powerful tool in molecular biology. This specific and sensitive method enhances gene probe detection of gene sequences by amplifying the target sequence using primers specific for a particular organism or group of organisms. This method increases the chance of detecting rare sequences in a mixture of heterologous DNA (Atlas *et al.*, 1992), so small soil samples (or any other samples) are sufficient. Because DNA samples might not be completely pure, dilutions can be performed to prevent inhibitory effects of humic substances on PCR, even if it limits the sensitivity of this method (Moré *et al.*, 1994).

Following PCR, fingerprints may be generated to screen microbial diversity in soil (Porteus *et al.*, 1997), or the amplified product may be directly used to detect, isolate, and monitor organisms which contain genes that encode important metabolic pathways, such as pollutant-degrading bacteria, as reported by Greer *et al.* (1993), or to monitor genetically-modified microorganisms (Cullen and Hirsch, 1998). The sensitivity of these kinds of studies has been improved with the PCR technology as compared to DNA hybridization (Cullen and Hirsch, 1998).

Use of PCR and specific gene markers are very helpful to identify organisms and genotypes where morphology does not give enough information, such as mycorrhizae, which is a symbiotic association between a fungus and the root system of a host plant (Bachmann, 1994). The targeted DNA has to be specifically amplified from a mixture of plant, bacterial, and fungal DNA. The amplified product may then be analysed by RFLP (restriction fragment length polymorphisms), sequencing or oligonucleotide probing (Gardes and Bruns, 1993).

2.2 Research Objective

The objective of this study was to develop an environmentally friendly (e.g., using as few and as harmless reagents as possible), simple, rapid, and reliable method for obtaining pure, high-molecular-weight DNA from different Canadian soil orders for PCR amplification applications. Thirteen methods of direct DNA extraction were tested on four different soil orders to determine their efficiency to yield DNA of quality suitable for PCR amplification. The four types of soil were chosen to represent a wide range of soil characteristics because the method of DNA extraction to be developed had to yield DNA of suitable purity for PCR amplification from very different soil types. The relationships between soil physical and chemical properties with DNA yield, quality, and PCR amplification success were further investigated.

2.3 Materials and Methods

2.3.1 Site characteristics and sample collection

Four different soil orders (The Canadian System of Soil Classification, 1998) were collected in Northern Alberta during summer 1999 with a soil core sampler. On each site, each representing one soil order, five soil cores were collected within an area of 2 m^2 at about the same distance from each other to avoid big variations in soil characteristics. Precautions were taken to disturb as little as possible the different soil horizons. Soil cores were slid into PVC tubes of about 20 cm long and 6 cm of diameter and transported to the University of Alberta, where they were stored at –20°C prior to processing. Each sample was subdivided into the organic and mineral soil horizons, sieved through a 4.75 mm sieve, air-dried for 3 to 7 days, and kept in plastic bags. For the Typic Fibrisol soil, the soil particles were manually reduced in size by rubbing them between the hands and were then passed through a 4.75 mm sieve. For DNA extraction, the five samples were used but for the soil physical and chemical analyses, two or three composite soil samples were analysed (the five samples were not analysed separately to reduce the cost of the

experiment). Prepared samples were put in plastic bags and stored at 4°C until analysed (between one and eighteen months).

2.3.1.1 Site 1

The first site was located in the Edson area, at the location North East (N.E.)-¼ section (Sec.) 17, Township (Tp.) 52, Range (R.) 18, West 5th Meridian (W.5) (N.E. Sec.17, Tp.52, R.18, W.5) on the soil map of Edson area (83-F, East-½). It was an Eluviated Eutric Brunisol (EEB) soil of the Heart Soil Association, referred to as Degraded Eutric Brunisol (mapping unit: HTR 1) in the Soil Survey and Land Evaluation of the Hinton-Edson area, Alberta (Dumansky *et al.*, 1972), that was developed on an aeolian sandy material with distinct FH and Ae horizons. It is described as very coarse, rapidly drained, mixed, acid to neutral, weakly calcareous, and cold-humid (Dumansky *et al.*, 1972). Possible minor soil inclusions were Orthic Regosol and/or Orthic Podzol (Dumansky *et al.*, 1972). The stand was dominated by aspen (*Populus tremuloides* Michx.) mixed with white spruce (*Picea glauca* Moench) and lodgepole pine (*Pinus contorta* Dougl.). Shrub species consisted of *Vaccinium* spp. and green alder (*Alnus crispa* [Ait.]), and a herbaceous stratum of mosses and *Lycopodium* spp.

2.3.1.2 Site 2

The second site was also located in the Edson area, at the Taki Experiments site around N.E. Sec.18, Tp.50, R.16, W.5 on the same map (RGE, Rd 181A: intersection highway 16 East). The soil at this site was a Brunisolic Grey Luvisol (BGL) of the Hanlan series of the Marlboro Soil Association that was developed on till materials and referred to as an Orthic Grey Luvisol (Mapping unit: MLB 5), with distinct FH, Ae, and Bt horizons. It was a significant group within the dominant soil referred to as Bisequa Grey Luvisol (Marlboro association, Wildhay series, mapping units: MLB 6). It is described as moderately fine, moderately well drained, mixed, acid to neutral, and cold-humid (Dumansky *et al.*, 1972). The stand, dominated by lodgepole pine, regenerated after a fire, which occurred in 1956.

2.3.1.3 Site 3

The third site was located close to the Ellerslie Research Station, University of Alberta (N.E. ¼, Sec.24, Tp.51, R.25, W.4). At this site the soil was a moderately to imperfectly drained Gleyed Eluviated Black Chernozem (GEBC) with distinct FH and Ah horizons (Sanborn, 1981). It was a white spruce dominated stand with a few balsam poplars (*Populus balsamifera* L.) and aspens (Sanborn, 1981).

2.3.1.4 Site 4

The fourth site was located at the Syncrude Canada Ltd. plant north of Fort McMurray, Alberta (N 57° 03.50' 53'', W 111° 39' 284'' GPS [Global Positioning System]; Sec.15, Tp.94, R.111). The soil there was a Typic Fibrisol (TF) (organic) with distinct Of and Om horizons. Samples were taken next to a ditch with blossoming spruces and willows. Fibrisol soils are composed largely of relatively undecomposed fibric organic material, which occur extensively in Canada, particularly in peat deposits dominated by *Sphagnum* mosses.

2.3.2 Physical and chemical soil analyses

2.3.2.1. pH measurement

Before sub-samples were randomly taken from each bag (each containing the soil from one horizon), the soil was thoroughly mixed. Soil pH was determined following procedures outlined in Kalra and Maynard (1991). Between 0.52 g and 5.00 g of soil (depending on the soil density) was weighed and put in a paper cup. A ratio in volume (vol) of 1:2 soil-to-liquid mixture was used (1:4 for organic soils is often used) (Kalra and Maynard, 1991) but in some cases, more liquid was added to obtain enough supernatant to measure the pH. The pH was measured in dH_2O (data not shown) and in a solution of 10 mM $CaCl_2$. After the liquid was added to the soil, the mixture was stirred three times during 30 min, then the pH was measured with a Fisher Accumet® pH meter Model 630. Duplicates of some samples were measured to determine within-run precision (Kalra and Maynard, 1991). Results of duplicates were very similar; thus, the within-run precision of the method was good. The average of the duplicates was used in the

analyses. A standard (a soil from the Ellerslie Station) with a pH of 6.20 in H_2O was included in the analyses to evaluate the validity of results.

All the statistical analyses were done with the pH-measurement in 10 mM $CaCl_2$. Lower pH-values are obtained in a solution of $CaCl_2$ compared to measurements done in H_2O due to high exchange acidity (Nykvist and Skyllberg, 1989). However, when a solution of 10 mM of $CaCl_2$ is used instead of water, small differences in salt contents are masked without displacement of large fractions of the H^+ or Al^{3+} ions, and it minimizes liquid junction potential effect (McLean, 1982). Moreover, pH is almost independent over a wide range of soil/$CaCl_2$ ratios, values are more reproducible, good approximations of soil pH under field conditions are obtained, results are not as dependent on the position of the electrodes, and the suspension effect is eliminated by maintaining soil in a flocculated condition (Kalra and Maynard, 1991). However, it has to be remembered that pH-values measured in $CaCl_2$ will be approximately 0.5 pH units lower than in dH_2O (Juma, 1999). When the pH is measured in water, the dilution factor is more important, but it is closer to the true pH of a soil solution having low salt concentration in solution (Juma, 1999).

It has been shown that with a sufficient sample number, the pH-meter gives values that form a normal distribution with standard deviations that do not follow mean values. This is not always the case with hydrogen-ion concentration. Thus, the pH-meter values were used directly in the statistical analyses without converting them to hydrogen-ion concentration.

2.3.2.2. Total nitrogen, organic carbon, and organic matter content

Again, before the sub-samples were randomly taken, the bag of soil was thoroughly mixed. The percentages of organic carbon and of total nitrogen of the samples were analysed with a Carlo-Erba NA 1500 series 2, Nitrogen/Carbon/Sulphur automated analyser (Milan, Italy; Strumentazione) and quantified using procedure of Baccanti and Colombo (1992). First, a few grams of each sample were ground 3 min at maximum speed with a Retsch Brinkmann MM2 Vibrator and dried at 60°C overnight. Then, 20 to 30 mg of the sub-sample was acidified with 50 µl of 6 M HCl and oven dried at 70°C

before the capsule was closed. Organic carbon was determined by dry combustion, with carbon content expressed relative to the original soil weight (Baccanti and Colombo, 1992). Duplicates were also analysed for some samples. Acetanalid (10-36% nitrogen, 71-09% carbon) was used as a reference and a Dark Brown Lethbridge soil with 0.202% nitrogen and 2.065% organic carbon was used as an internal standard. The standard deviations obtained for the standard were 0.002646 for the percentage of total nitrogen, and 0.013077 for the percentage of organic carbon. The percentage of organic matter (%om) was calculated using the equation (Kalra and Maynard, 1991):

percentage of organic matter = percentages of organic carbon x 1.724

The Van Bemmelen coefficient of 1.724 came from the fact that organic matter is composed of approximately 58% of organic carbon. However, this coefficient varies from one soil to another, so the values of the percentage of organic matter (presented later in Table 2.2) are only estimates of the real amount present in the soil samples (Kalra and Maynard, 1991).

2.3.2.3. Humus fraction content

Conventional humic acids (HA-A), clay-associated humic acids (HA-B), and humin were determined according to Anderson and Schoenau (1993). First, air-dried soils were sieved through a 2 mm screen, and 15 g of each soil was put into 250 ml plastic centrifuge bottles. Samples were then mixed with 150 ml of 0.5 M HCl and set aside for 1 h, but were occasionally stirred (this step may remove a small amount of organic matter). Samples were then centrifuged for 15 min at 9000 x g, and the supernatants were discarded. This step was a pre-treatment to remove floating plant debris and inorganic forms of C, N, P, and S prior to extraction with NaOH. To remove any remaining HCl, 150 ml of dH_2O was added to the bottles, which were mixed and then centrifuged at 9000 x g for 15 min. Supernatants were again discarded. For the alkali extraction, 150 ml of freshly made 0.5 M NaOH was added to the bottles and oxygen-free N_2 gas was used to flush the head space to reduce oxidation and CO_2 absorption. Caps were quickly tightened and the bottles were placed for 18 h on an end-over-end shaker at 60 rev min^{-1}.

To separate the NaOH extract, that is, the HA and the fulvic acid (FA) fractions, from the soil residue (humin fraction), bottles were centrifuged 15 min at 9000 x g. Supernatants were carefully decanted into clean centrifuge bottles for further separation into HA-A and FA-A fractions, and the humin portion was oven dried overnight at 70°C and weighed. A solution of 6 M HCl was added to the NaOH extract with a burette until a pH of 1.5 was reached. This led to the precipitation of a portion of the organic matter. The dark brown precipitate was the HA-A portion, while the part that remained dissolved in solution was the FA-A fraction, which was not quantified. The two fractions were separated by centrifugation at 9000 x g for 15 min. The HA-A portion was oven dried overnight at 70°C and then weighed. Finally, the oven dried humin portion was mixed with 150 ml of dH_2O, sonicated for 10 min with an ultrasonifer at 125 W, and then allowed to settle for 48 h. Bottles containing the humin portion were centrifuged at 9000 x g for 15 min, and the supernatants were removed and acidified to a pH of 1.5, as described above. The HA-B precipitates were separated from the FA-B fractions that remained in solution by centrifugation at 9000 x g for 15 min. The HA-B portions were then oven dried overnight at 70°C and weighed.

2.3.2.4. Texture

Soil texture was determined following the hydrometer method (with pretreatment to remove organic matter and soluble salts) outlined in Kalra and Maynard (1991). The hydrometer method depends fundamentally upon Stoke's Law, in which the sedimentation parameter is a function of the hydrometer settling depth, the solution viscosity, and the particle and solution density. The soil particles are considered to have a volumic density of 2.65 g l^{-1} (Kalra and Maynard, 1991).

Fifty g of soil was transferred to a 1-L beaker into which 50 ml of dH_2O was added. Fifty millilitres of 30% H_2O_2 was slowly added to the beaker, 10 ml at a time to prevent frothing. The soil suspension was mixed and the beaker was covered to prevent clay projections. The reaction was checked for about 20 min. When frothing was excessive, the beaker was cooled in cold water. The cover was rinsed to wash the soil particles into the beaker. This was done until the 50 ml of H_2O_2 was added. The beaker was heated to

about 80°C when frothing has ceased. More H_2O_2 was added and used to rinse the sides and the cover of the beaker until the addition of H_2O_2 did not produce any frothing and the sample had turned to a bleached colour. The volume was brought to 400 ml with dH_2O and the solution was boiled for 1 h to destroy excess peroxide. The beaker was then left at room temperature to cool down and settle the suspension. The supernatant was discarded using a pipette and then 250 ml of H_2O was added vigorously to disperse the sediment and the supernatant was again discarded. This step was repeated twice. The presence of salt was checked by testing with $AgNO_3$ for Cl^- and with $BaCl_2$ for SO_4^{2-}.

The suspension was then transferred into the metal dispersing cup and the volume was adjusted to 400 ml with H_2O. Fifty millilitres of calgon solution (100 g l^{-1}) (hexametaphosphate plus enough Na_2CO_3 to bring the pH to about 8.3) was added. The solution was mixed 15 min with the electric stirrer (malted milk mixer type). The suspension was transferred to a sedimentation cylinder (1-L mark at 36 ± 2 cm from the bottom). The volume was adjusted to 1 L with dH_2O. The cylinder was covered and allowed to settle overnight to equilibrate thermally to temperature. The sediment was dislodged from the bottom using strong upward strokes of a plunger for about 2 min. Then, two or three slow, smooth strokes were done to finish stirring. The plunger was removed and the hydrometer was immediately lowered into the suspension and the reading was taken exactly 40 s after the end of the stirring. Two drops of amyl alcohol were added if the suspension was frothing. The hydrometer was removed and the temperature was recorded at a depth of about 5 cm. The cylinder was allowed to settle and the hydrometer and the temperature readings were repeated at the end of the second hour. A similar protocol was followed for the blank, which consisted of 50 ml calgon solution and 950 ml dH_2O. The suspension was then transferred through a 0.05 mm sieve and the sediment was washed until the flow-through water was clear. The sand was transferred into a tared 250 ml beaker. The excess water was evaporated at room temperature, after which the sand was dried at 105°C for 24 h. The beaker was cooled down in a desiccator and weighed.

Calculation: For each degree above or under 20°C, 0.36 graduation was added or subtracted from the hydrometer readings. The readings were also corrected by subtracting the value obtained for the blank. The percentage of sand, silt, and clay were calculated as follows:

(a) %silt + %clay = (corrected reading after 40 s/weight of sample [g]) x 100

(b) %clay = (corrected reading after 2 h/weight of sample [g]) x 100

(c) %silt = (a) – (b)

(d) %sand = (weight of sand [g]/weight of sample [g]) x 100

where weight of sample (g) = corrected reading after 40 s + weight of sand (g).

2.3.3 DNA extraction

In each of the 13 methods tested, the lysing step was a combination of physical (bead-beating and heat shocking) and chemical (100 mM NaCl, 500 mM Tris-HCl pH 8.0, 10% SDS buffer and/or AP1 buffer) methods. Bead-beating was preferred to freezing-thawing because it has been shown to be more efficient in lysing cells (e.g., Moré *et al.*, 1994). The SDS-based cell lysis protocol was found to provide higher yield of DNA in comparison with freezing-thawing and Sarkosyl-based lysis protocols (Trevors *et al.*, 1992 - as described by Zhou *et al.*, 1996), and the combination of SDS with bead mill homogenization was found to result in higher cell lysis efficiency for *Bacillus* endospores, but might leave the smallest cells intact (Moré *et al.*, 1994). The combinations of extraction method and soil types performed in this study are presented in Table 2.1.

Sometimes, when a method was unsuccessful for one soil type, or if it took longer or was not as efficient as another method with the same soil type, it was not tried with the other soil types. For example, no trace of DNA could be seen on the agarose gel after electrophoresis of the extract obtained when the crude DNA extract from two of the

horizons of the EEB soil type was purified with method 6, and no PCR product was obtained. Therefore, this method was not tested with any other soil samples.

Table 2.1 Combinations of soil type and method of direct DNA extraction tested.

Soil type	Horizon (Sample)	Method of DNA extraction[a]												
		1	2	3	4	5	6	7	8	9	10	11	12	13
EEB	FH	X	X		X	X		X	X				X	X
	FH(4)	X	X		X	X	X	X	X				X	X
	Ae	X			X	X		X	X				X	X
	Ae(5)	X			X	X	X	X	X				X	X
BGL	FH	X		X	X	X		X	X				X	X
	Ae	X			X			X	X				X	X
	Bt	X			X			X					X	X
GEBC	FH	X			X	X		X					X	X
	Ah	X			X	X		X					X	X
TF	Of	X			X	X		X		X	X	X	X	X
	Om	X			X	X		X		X	X	X	X	X

[a]X: method tested

2.3.3.1 Method 1

A modified DNeasy Plant Mini Kit (QIAGEN Inc., Mississauga, Ontario) method was used for method 1. Five replicates of between 0.1 and 0.5 g of soil (depending on the soil density) were mixed with 1.0 ml of 100 mM sodium phosphate buffer (100 mM Na_2HPO_4, 100 mM NaH_2PO_4, pH 8.0) and 0.5 ml of lysing buffer (100 mM NaCl, 500 mM Tris-HCl pH 8.0, 10% SDS) in a 2-ml screw cap polypropylene microcentrifuge tube containing 0.5 g of 0.5 mm diameter glass beads (Biospec Products, Bartlesville, Oklahoma). Cells were homogenized with a Mini-Beadbeater (Biospec Products) for 3 min (4 min for the Typic Fibrisol soil). Samples were then centrifuged 3 min at 12000 rev min^{-1}. Samples were then processed according to the DNeasy Plant Mini Kit procedure. That is, aliquots were put into new tubes in which 4 µl of RNase A and 400 µl of AP1 buffer were added. They were incubated 15 min at 65°C (heat shock) during which the tubes were shaken a few times. Then, 130 µl of AP2 buffer was added and the tubes were shaken and put on ice for 5 min. Each thick lysate mixture was put into a QIAshredder™ spin column (filtration and homogenization) contained in a 2 ml collecting tube. The

samples were centrifuged 2 min at maximum speed and the flow-through fractions were transferred to new tubes into which a ½ vol of AP3 buffer and 1 vol of 100% ethanol were gently mixed. Next, 650 µl of this mixture was transferred into DNeasy columns and centrifuged 1 min at 8000 rev min^{-1}. The flow-through portions were discarded and the above step was repeated for the remainder of the samples. The DNeasy columns were put into new 2 ml tubes and 500 µl of AW buffer was added. Columns were centrifuged 1 min at 8000 rev min^{-1} and the flow-through fractions discarded. Another 500 µl of AW buffer was added and the tubes were centrifuged 2 min at maximum speed to dry the membrane. The columns were transferred again into new centrifuge tubes before 100 µl of preheated (65°C) AE buffer was added. Columns were centrifuged 1 min at 8000 rev min^{-1} to elute the DNA sample. A second 100 µl of preheated AE buffer was added followed by centrifugation.

2.3.3.2 Method 2

Two-hundred and fifty milligrams of organic soil horizon and 500 mg of mineral soil horizon were mixed with 1 ml of AP1 buffer. Subsequently, 8.33% (wt/vol) PVPP, 0.5 ml of sodium phosphate buffer and 0.35 g of glass beads were added to the mixture before homogenization for 3 min in the Mini-Beadbeater. Ten millilitres of RNase A was added before incubation for 1 h at 65°C. After 3 min centrifugation at 12000 rev min^{-1}, supernatants were mixed with 130 µl of AP2 buffer. Subsequent steps followed method 1, begginning with the QIAshredder™ spin column.

2.3.3.3 Method 3

This method was identical to method 2 with the exception of the lysing steps, were 750 µl of lysis buffer (100 mM NaCl, 500 mM Tris-HCl pH 8.0, 10% SDS, 8.33% PVPP) and 750 µl of 0.1 M sodium phosphate buffer were added to tubes containing 0.25 g of the FH soil horizon from site 2 (BGL soil) and 0.35 g of glass beads. Samples were homogenized 3 min in the Mini-Beadbeater. The subsequent steps were similar to method 2.

2.3.3.4 Method 4

Between 0.075 and 0.5 g of soil (depending on the soil density) were used. The DNeasy Plant Mini Kit procedure was used without modification except for an additional 3 min centrifugation at 12000 rev min^{-1} after the 1 h incubation at 65°C.

2.3.3.5 Method 5

It was the same as method 4 except that 8.33% of PVPP was added to the AP1 buffer.

2.3.3.6 Method 6

Either 0.25 g of FH soil horizon or 0.50 g of Ae soil horizon from site 1 (EEB soil) was mixed with 0.35 g of glass beads, 750 µl of lysis buffer (100 mM NaCl, 500 mM Tris-HCl pH 8.0, 10% SDS), and 500 µl of sodium phosphate buffer. Samples were homogenized 3 min in the Mini-Beadbeater. Four microlitres of RNase A was added to the homogenate and incubated for 1 h at 65°C. Tubes were then centrifuged for 3 min at 12000 rev min^{-1} and an extraction with 500 µl of phenol:chloroform:isoamyl alcohol (vol:vol:vol/25:24:1) was performed on the supernatants. The upper phase containing DNA was purified with magnetic beads (Dynabeads® DNA DIRECT™, Dynal A.S. Oslo, Norway) (Rudi *et al.*, 1997).

2.3.3.7 Method 7

Between 0.08 and 0.50 g of soil was used with 0.35 g of glass beads, 1 ml of sodium phosphate buffer, and 0.5 ml of lysis buffer (100 mM NaCl, 500 mM Tris-HCl pH 8.0, 10% SDS) buffer. After 3 min homogenization in the Bead-Beadbeater, RNase A treatment and centrifugation were done as in method 6. Ten microlitres of the supernatant was loaded in a 1% agarose gel prepared with 1X TAE buffer (40 mM Tris-acetate, 1 mM EDTA [ethylenediaminetetraacetate] pH 8.0) and run between 120 V and 150 V for about 2 h. Gel was stained in EtBr and destained in dH_2O. Gel cores were then taken out using pipette tips with cut end (the tips were cut approximately 3 mm from their end to facilitate the entry of the gel in the tip) up and down the visible brown band left by phenolic contaminants. They were put in eppendorf tubes containing 500 µl of double

dH_2O (ddH_2O) and were melted at 70°C. An aliquot of 25 µl of these extracts was used for the PCR reaction.

2.3.3.8 Method 8

Method 8 was the same as method 7 except that 10% PVP was added to the 1% agarose gel and gel cores were put in 450 µl of ddH_2O instead of 500 µl. When PVP is added to the gel, the loading dye bromophenol blue migrates slower than the DNA because it contains phenolic compounds. A non-phenolic loading dye such as xylene cyanol (Young *et al.*, 1993) can be used to follow DNA migration in this case, but it was not used in this study. Young *et al.* (1993) did not find any apparent difference in the purification performance between gels prepared with TAE or TBE (89 mM Tris-borate, 2 mM EDTA pH 8.0) buffer; therefore, only TAE buffer was used in this study.

2.3.3.9 Method 9

Method 9 was the same as method 8 except that 0.45% PVP was added to the 1% agarose gel instead of 10% PVP. Young *et al.* (1993) have reported that the addition of PVP did not reduce the yield of PCR product until PVP concentration was equal to or greater than 0.5% (wt/vol). Because it was difficult to estimate the concentration of PVP after dilution of the gel core in ddH_2O and then in the PCR reaction, a concentration of PVP of less than 0.5% was used to make sure that it did not inhibit the PCR reaction.

2.3.3.10 Method 10

Method 10 was the same as method 9 but a phenol:chloroform:isoamyl alcohol (vol:vol:vol/25:24:1) extraction was performed on the samples before they were loaded into a 1% agarose into which 2% PVP was incorporated.

2.3.3.11 Method 11

Method 11 was the same as method 7 until the centrifugation steps. Samples were then mixed with about 0.5 g of PVPP, vortexed, incubated at 65°C for 10 min, vortexed again and centrifuged 3 min at 12000 rev min^{-1}. Supernatants were mixed with 0.4 vol of 7.5 M ammonium acetate (NH_4OAc), placed 10 min on ice and centrifuged 3 min at 12000 rev

min^{-1}. An aliquot of 150 µl of supernatant was poured into Sephacryl S-400 HR MicroSpin™ Columns (Amersham Pharmacia Biotech Inc.) and centrifuged at 3000 rev min^{-1} for 2 min following the procedure A of General Protocol of MicroSpin™ Columns Instructions.

2.3.3.12 Method 12

Method 12 was the same as method 11 except that the PVPP step was not done. Between 0.085 and 0.50 g of soil was used, depending on the soil density.

2.3.3.13 Method 13

Method 13 was adapted from the method described by Berthelet *et al.* (1996), but the purification step using acid-washed PVPP was replaced by non-acid-washed PVPP. Acidified PVPP spin columns are more efficient for binding humic substances but they were shown to degrade DNA (Cullen and Hirsch, 1998). This method was similar to method 11 but the Sephacryl S-400 HR resin was replaced by a slurry of PVPP and 20 mM potassium phosphate buffer (20 mM K_2HPO_4, 20 mM KH_2PO_4).

For methods 12 and 13, when there was no amplification, extracts were passed into new column(s) until successful amplification was obtained. Columns can be reused after being washed in a boiling diluted solution of SDS and rinsed 10 times with sterile dH_2O.

2.3.4 Determination of DNA quantity and purity

DNA purity was determined with a UV/visible spectrophotometer (Perkin-Elmer, Lambda 3) at 230 nm (A_{230}), 260 nm (A_{260}), and 280 nm (A_{280}). To be accurate, the concentration of the sample was adjusted so that the absorbency readings fell between 0.1 and 1.0. A ratio of $A_{260}/A_{280} < 1.7$ indicated the presence of protein contamination and a ratio of $A_{260}/A_{230} < 2.0$ indicated the presence of humic compound contamination (Cullen and Hirsch, 1998). Quantification of the concentration (yield) of DNA was determined at A_{260} using a spectrophotometer (an absorbency of 1 = 50 µg of DNA ml^{-1}) (Sambrook *et al.*, 1989). The yield was calculated using the value of the A_{260}, the dilution factor and the

amount of soil used in the extraction procedure. Alternatively, DNA yield and size were also estimated by comparison of replicate samples with a Low DNA Mass™ Ladder and a 1 kb DNA Ladder (GIBCO-BRL®, Life Technologies) on a 1% agarose gel stained with EtBr (0.4 mg l^{-1} dH_2O), de-stained in dH_2O and photographed with Polaroid® film (Polapan 667 instant film) under UV illumination (312 nm). In this case, 10 µl of extracted DNA, 2 and 4 µl of Low DNA Mass™ Ladder, and 5 µl of 1 kb DNA Ladder were respectively electrophorised in an agarose gel prepared with 1X TAE buffer (40 mM Tris-acetate, 1 mM EDTA pH 8.0) or 1X TBE buffer pH 8.4 (89 mM Tris-borate, 2 mM EDTA pH 8.0). Electrophoresis of 4 µl of Low DNA Mass Ladder results in bands containing 200 (2000 bp), 120 (1200 bp), 80 (800 bp), 40 (400 bp), 20 (200 bp), and 10 (100 bp) ng of DNA respectively (GIBCO-BRL Products & Reference Guide 2000-2001, p.17-12).

2.3.5 DNA extraction for PCR positive control

Fruiting bodies from the ectomycorrhizal fungus *Laccaria bicolor* from Bonnyville Forest Nursery were ground in liquid nitrogen until a fine powder was obtained. DNA was then extracted using the DNeasy Plant Mini Kit procedure.

2.3.6 PCR amplification

DNA concentration was adjusted for use in PCR amplification with three or four serial dilutions with ddH_2O (1/25, 1/150, 1/450, 1/1000). These DNA templates were amplified using the primers ITS1-F (fungus specific: 5-CTTGGTCATTTAGAGGAAGTAA-3) and ITS4 (universal: 5- TCCTCCGCTTATTGATATGC-3) which amplify the entire ITS region from most basidiomycetes and ascomycetes as well as a small amount of plant materials (Gardes and Bruns, 1993), giving PCR products of about 700-800 bp. The master mix volume was 25 µl containing 20 mM Tris pH 8.4, 5 mM $MgCl_2$, 100 mM KCl, 2.5% Triton X-100, 0.7 U of *Taq* polymerase, 400 µM of dNTPs (Amersham Pharmacia Biotech Inc.), and 1 µM of each primer (GIBCO-BRL®, Life Technologies). This mixture was covered with approximately 25 µl of mineral oil, and 25 µl of different

dilutions of the DNA extracts was added to the reaction mixture when the temperature reached 94°C. Positive (*Laccaria bicolor* DNA) and negative (no DNA added) controls were included. For the methods using gel purification (methods 7 through 10), a core taken from the periphery of the purification gel (where no DNA has been loaded) was also used as a blank in the PCR amplification reaction. A pre-incubation step at 94°C for 2 min was followed by 30 cycles (94°C for 1 min, 55°C for 1 min and 72°C for 2 min). A final extension cycle of 10 min at 72°C was added. PCR fragments were visualized on a 1% agarose gel as described above. The amplification success was assessed using an ordinal scale: 0 (no amplification), 1 (weak amplification), 2 (good amplification), and 3 (excellent amplification).

2.3.7 Statistical analyses

The experimental design was a randomized block design with the following mathematical model:

$$Y_{ijkl} = \mu + A_i + B_j + C_{k(j)} + AB_{ij} + E_{(ijk)},$$

where μ is the overall population mean, A_i is the effect of the i^{th} method of direct DNA extraction ($i = 1\ldots5$) with 4 degrees of freedom (DF), B_j is the effect of the j^{th} soil type or block ($j = 1\ldots4$) with 3 DF, $C_{k(j)}$ is the effect of the k^{th} soil horizon ($k = 1\ldots2$ or 3) within the j^{th} soil type with 5 DF (one DF for the EEB, the GEBC, and the TF soils and 2 DF for the BGL soil), AB_{ij} is the interaction between DNA extraction method and soil type with 12 DF, which is also the error term for testing the effect of method, and $E_{(ijk)}$ is the residual and is equivalent to the interaction between method and soil horizon within soil type.

The main interest of this study was to find the DNA extraction method with the highest efficiency with a variety of soil types regarding its capacity to yield DNA suitable for PCR amplification. Therefore, DNA extraction method was considered a fixed factor, and each site or block (representing a different soil type) and horizon nested within soil type

were random factors. Because of the large amount of missing data, the analyses were performed on only five methods (methods 1, 4, 5, 12 and 13). Normality and homoscedasticity of data were tested using the SAS Univariate procedure, and the Brown-Forsythe test using SAS for Windows (SAS 1999, version 8.1). DNA yield data needed log-transformation and two extreme values of A_{260}/A_{230} ratio were excluded from the analyses to fulfil these two assumptions for ANOVA. Analysis of variance was conducted using the MIXED procedure for the variables (using the average of the five replicates of each horizon) of A_{260}/A_{230} and A_{260}/A_{280} ratios, the log(DNA yield), and the PCR amplification success (PCR = 0, 1, 2, or 3). A multiple regression analysis was performed to study the impact of the physical and chemical soil properties (independent variables) on all the dependent variables (using the average of the five samples of each horizon) using the REG procedure and forward stepwise selection with $\alpha = 0.15$. First, the GLM procedure was used to test if there was an interaction between "method of DNA extraction" and the soil characteristics according to the Type III SS (Sum of Squares). When at least one of the interactions was significant at the 0.05 level, the REG procedure was performed for each method separately. When no interaction was significant, the REG procedure was used on the average of all methods together. The percentage of organic matter was excluded from the analysis, because it was calculated using the percentage of organic carbon; thus, the two variables were highly correlated.

The two null hypotheses (H_0) tested were:

1) The main factor "method of DNA extraction" had no effect on the dependent variables (A_{260}/A_{230} ratio, A_{260}/A_{280} ratio, log[DNA yield], and PCR amplification success), and

2) The independent variables (the soil characteristics) were not correlated with the dependent variables.

2.4. Results

2.4.1 Physical and chemical soil analyses

The means of the five replicates for each soil horizon of each soil type for the physical and chemical analyses are presented in Table 2.2.

Table 2.2 Averages of the soil chemical and physical properties of the five replicates for each soil horizon of each soil type.

		Chemical properties							Physical properties			
Soil type	Horizon	pH[a]	% tn	% oc	% om	% humin	% HA-A	% HA-B	%clay <2μm	%silt 2-50μm	%sand >50μm	Texture class
EEB	FH	5.50	0.99	21.09	36.30	77.64	8.59	2.13	7.30	13.25	79.46	Loamy sand
	Ae	4.81	0.09	1.52	2.62	84.17	1.27	0.66	7.37	13.64	79.99	Loamy sand
BGL	FH	3.75	0.75	23.65	40.77	68.97	8.19	3.12	12.76	47.87	39.36	Loam
	Ae	4.10	0.12	1.66	2.86	88.36	0.72	0.71	8.70	66.56	24.75	Silt Loam
	Bt	4.71	0.10	1.31	2.26	87.81	0.56	0.81	8.13	42.94	48.93	Loam
GEBC	FH	5.23	1.43	32.16	55.44	57.57	20.63	5.80	13.68	50.71	35.61	Silt Loam
	Ah	5.86	1.20	21.36	36.82	61.12	17.35	3.49	19.10	41.14	39.76	Loam
TF	Of	7.09	0.78	44.47	76.66	69.46	0.60	0.55	ND[b]	ND	ND	ND
	Om	5.60	0.95	37.82	65.21	67.24	10.32	5.00	9.10	14.60	76.30	Sandy Loam

[a] Measured in a solution of 10 mM $CaCl_2$
[b] ND: not determined

All four soil types studied were acidic except for the Of horizon of the TF soil, which was neutral (pH 7.09). On average, the BGL was the most acidic soil, especially the FH horizon (pH 3.75), followed by the EEB and the GEBC soil types. The TF soil was the least acidic.

The percentage of total nitrogen ranged from 0.09% for the Ae horizon of the EEB soil to 1.43% for the FH horizon of the GEBC soil. It usually decreased with depth for all soil types except for the TF soil. The GEBC soil had the highest total nitrogen content for both horizons, and the mineral horizons of the BGL soil (Ae and Bt) and of the EEB soil (Ae) had the smallest amount of total nitrogen.

The percentage of organic carbon decreased with soil depth for all soil types. The values ranged between 1.31% for the Bt horizon of the BGL soil and 44.46% for the Of horizon of the TF soil. On average, the organic soil (TF) had the highest organic carbon content and the EEB the smallest amount. The percentage of organic matter closely followed the same pattern as the percentage of organic carbon. The Bt horizon had the smallest quantity with 2.26% and the Of horizon the highest content with 76.66%.

The percentage of humin was always smaller for the top soil horizon and ranged from 57.57% for the FH horizon of the GEBC soil to 88.36% for the Ae horizon of the BGL soil. The GEBC soil had the smallest values for both horizons and on average, the BGL soil had the highest percentage of humin.

As for the percentage of total nitrogen, the percentage of HA-A declined with soil depth except for the TF soil. The Bt horizon had the smallest amount of HA-A with 0.56%, and the FH horizon of the GEBC soil had the highest amount with 20.63%, followed by its Ah horizon (17.35%). On average, the BGL soil type had the smallest percentage of HA-A, and the GEBC the highest amount. The percentage of HA-B varied between 0.55 for the Of horizon to 5.80 for the FH horizon of the GEBC soil, which had the highest values for both horizons.

The texture class was determined following the soil texture classes triangle from the Canadian System of Soil Classification (1998) using the percentage of clay and sand. The EEB soil had the smallest amount of clay (FH = 7.30% and Ae = 7.37%) and silt (FH = 13.25% and Ae = 13.64%) compared to the other soil types but had the highest percentage of sand (FH= 79.46%, Ae = 79.99%), which classified its two horizons as loamy sands. The FH and Bt horizons of the BGL soil and the Ah horizon of the GEBC soil were classified as loam, and the Ae horizon of the BGL soil and the FH horizon of the GEBC soil had the highest percentage of silt and the lowest percentage of sand, which classified them as silt loam. The two horizons of the GEBC soil had the highest percentage of clay. The physical properties had not been analysed on the Of horizon of

the TF soil because it was mainly composed of organic matter (76.66% om), but the Om horizon was classified as a sandy loam.

The variation in the results for the chemical and physical soil characteristics for the EEB, BGL, and GEBC soil types between the current study and those presented in Dumansky *et al.* (1972) and Sanborn (1981) might be due to small-scale variations in the landscape. Another possible explanation could be that the current study has been conducted decades later and soil conditions may have changed since then.

2.4.2 Soil DNA extraction

All the methods using the DNeasy Plant Mini Kit (methods 1 through 5) were time consuming, and with variable efficiency. The method using the magnetic beads (method 6) did not take as long, but no DNA was visible on the agarose gel after electrophoresis of the extracted DNA. Therefore, it was not tested on other soil types. The methods using agarose gel purification (methods 7 through 10) sometimes gave good amplification of DNA, but were also time consuming and required more manipulations with EtBr, which is a carcinogenic compound. The exposure of DNA to UV light may cause some mutations in the DNA strands that could lead to problems when the DNA has to be amplified or sequenced. Methods 11 and 12 were rather simple and quick and resulted in DNA extracts suitable for PCR amplification, but after comparing method 11 and method 12 on the TF soil, it appeared that the extra PVPP step in method 11 did not give better results than without it. Method 13 was a little bit longer than method 12 since the columns had to be washed and filled with PVPP prior to purification. Also, in method 13, some DNA extracts needed to be cleaned through more columns in order to be suitable for PCR amplification. On average, method 12 took between 2 and 3 h to process 10 soil samples from cell lysis to the end of the DNA purification steps, while method 13 took between 2.25 h to 3.50 h to process 10 samples. All other methods took longer or were less efficient.

2.4.3 Determination of DNA quantity and purity, and PCR amplification

The DNA strands obtained with every method, which all used the Mini-Beadbeater, ranged in size from 100 bp to 12 kb but most of the DNA was in the size range between 6 and 12 kb (data not shown). However, subsequent extraction of DNA from other soils with method 12 using the Mixer Mill MM 300 from QIAGEN with a 3 mm tungsten carbide bead instead of the Mini-Beadbeater and 0.5 mm glass beads yielded a higher concentration of DNA in the size range of about 12 kb, and less DNA of smaller size (see Chapter 3). Because the DNA extracted from soil gave a smear instead of well-defined bands, it was difficult to quantify the DNA with a mass ladder. Therefore, only the absorbency readings at 260 nm were used to determine DNA yield of the extracts. Only the results obtained with methods 1, 4, 5, 12, and 13 are presented because of missing data with the other methods. The purity and yield of DNA were not determined for method 6 because no DNA was visible on the agarose gel. They also had not been determined for the DNA extracted and purified with methods 7 through 10, because no attempt was made to purify the DNA from the gel. The results of the fixed effect "method of DNA extraction" of the ANOVA are presented in Table 2.3.

Table 2.3 Results of the ANOVA of the fixed effect "method of DNA extraction" for the A_{260}/A_{230} and A_{260}/A_{280} ratios, the log(DNA yield), and the PCR amplification success using the MIXED procedure with $\alpha = 0.05$.

Variable	F-value	Pr > F
A_{260}/A_{230}[a]	3.86	0.0307
A_{260}/A_{280}[b]	19.81	< 0.0001
Log(DNA yield)[c]	1.54	0.2524
PCR amplification[d]	23.59	< 0.0001

[a] Indication of humic acid contamination
[b] Indication of protein contamination
[c] DNA yield = µg DNA g^{-1} soil
[d] PCR = 0, 1, 2, or 3

The main effect "method of DNA extraction" had a significant effect on the A_{260}/A_{230} ratio at $\alpha = 0.05$. According to the A_{260}/A_{230} ratios, all the DNA extracts were contaminated to a certain degree with humic compounds since all the average ratios were smaller than 2 (Fig 2.1).

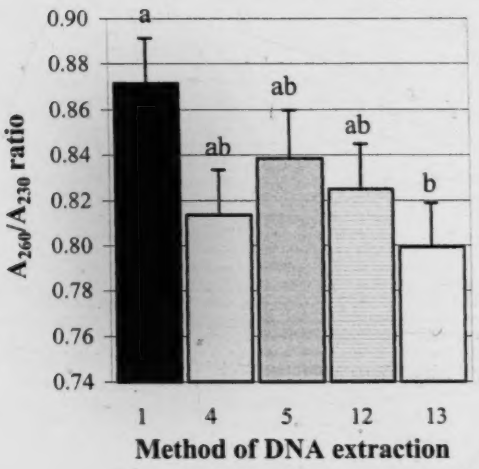

Fig. 2.1 Averages of the A_{260}/A_{230} ratios (indicator of humic substance contamination) for each method of direct DNA extraction. Values with no letters in common are significantly different ($\alpha = 0.05$) (Least Squares Means).

When averaging the A_{260}/A_{230} ratios for each method, method 1 yielded the smallest concentration of humic substances (highest ratio value) in the DNA extract, and method 13 the highest concentration. Only methods 1 and 13 were significantly different from each other.

The averages of the A_{260}/A_{230} ratios for each combination of method of DNA extraction and soil horizon are presented in Table 2.4. The values ranged from 0.731, when extracting DNA from the Of horizon of the TF soil type with method 13, to 0.941, when extracting DNA from the FH horizon of the EEB soil type with method 5.

Table 2.4 Averages of the A_{260}/A_{230} and the A_{260}/A_{280} ratios, the log(DNA yield), the DNA yield (μg DNA g^{-1} soil), and the PCR amplification success (between 0 and 3) for each combination of method of DNA extraction and soil horizon.

Observation	Method	Soil	Horizon	A_{260}/A_{230}	A_{260}/A_{280}	log(DNA yield)	DNA yield	PCR
1	1	EEB	FH	0.891	1.15	5.099	164	1.8
2	1	EEB	Ae	0.914	1.06	4.494	89.5	1.8
3	1	BGL	FH	0.860	1.10	7.229	1380	0.6
4	1	BGL	Ae	0.939	1.18	5.407	223	3.0
5	1	BGL	Bt	0.938	1.01	6.526	683	3.0
6	1	GEBC	FH	0.785	1.15	8.931	7560	0.0
7	1	GEBC	Ah	0.809	1.13	7.507	1820	0.0
8	1	TF	Of	0.860	1.32	6.494	661	1.0
9	1	TF	Om	0.853	1.28	5.961	388	1.2
10	4	EEB	FH	0.839	1.15	7.107	1220	0.0
11	4	EEB	Ae	0.828	1.18	5.983	397	0.0
12	4	BGL	FH	0.831	1.15	8.326	4130	0.0
13	4	BGL	Ae	0.812	1.18	6.229	507	0.0
14	4	BGL	Bt	0.796	1.38	5.011	150	0.8
15	4	GEBC	FH	0.763	1.16	9.250	10400	0.0
16	4	GEBC	Ah	0.775	1.19	8.261	3870	0.0
17	4	TF	Of	0.848	1.31	7.193	1330	0.0
18	4	TF	Om	0.831	1.13	7.340	1540	0.0
19	5	EEB	FH	0.941	1.17	6.996	402	0.0
20	5	EEB	Ae	0.860	1.05	4.796	121	0.6
21	5	BGL	FH	0.821	1.17	7.012	1110	0.0
22	5	GEBC	FH	0.784	1.16	8.503	4930	0.0
23	5	GEBC	Ah	0.806	1.20	7.908	2720	0.0
24	5	TF	Of	0.796	1.34	7.155	1280	0.0
25	5	TF	Om	0.860	1.13	7.626	2050	0.0
26	12	EEB	FH	0.779	1.26	7.237	1390	2.4
27	12	EEB	Ae	0.921	1.32	6.337	565	2.6
28	12	BGL	FH	0.820	1.28	8.055	3150	2.2
29	12	BGL	Ae	0.850	1.24	7.082	1190	2.4
30	12	BGL	Bt	0.797	1.44	6.466	643	3.0
31	12	GEBC	FH	0.837	1.23	7.466	1810	2.8
32	12	GEBC	Ah	0.796	1.33	7.162	1290	2.6
33	12	TF	Of	0.812	1.54	8.258	3860	2.8
34	12	TF	Om	0.814	1.36	7.696	2200	2.6
35	13	EEB	FH	0.792	1.36	7.293	1470	1.8
36	13	EEB	Ae	0.930	1.31	6.612	744	1.2
37	13	BGL	FH	0.796	1.31	7.600	2000	1.6
38	13	BGL	Ae	0.743	1.24	7.728	2270	1.4
39	13	BGL	Bt	0.797	1.50	6.466	643	3.0
40	13	GEBC	FH	0.808	1.36	7.402	1640	2.0
41	13	GEBC	Ah	0.782	1.37	7.139	1260	1.0
42	13	TF	Of	0.731	1.64	8.289	3980	1.0
43	13	TF	Om	0.811	1.38	7.678	2160	1.8

The main effect "method of DNA extraction" had a significant impact on the A_{260}/A_{280} ratio (indication of protein contamination) at $\alpha = 0.05$ (Table 2.3). According to the values of the A_{260}/A_{280} ratios, all the methods yielded DNA extracts contaminated by proteins because all the ratios were smaller than 1.7 (Fig. 2.2).

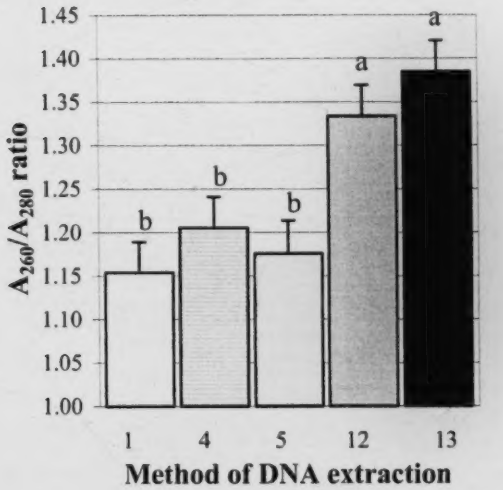

Fig. 2.2 Averages of the A_{260}/A_{280} ratios (indicator of protein contamination) for each method of direct DNA extraction. Values with no letters in common are significantly different ($\alpha = 0.05$) (Least Squares Means).

On average, method 13 yielded the smallest amount of protein in the DNA extracts (highest A_{260}/A_{280} ratio) whereas method 1 gave the highest amount of protein. Methods 12 and 13 had significantly higher ratios than methods 1, 4, and 5. The averages of the A_{260}/A_{280} ratios for each combination of method of DNA extraction and soil horizon are presented in Table 2.4. The values ranged from 1.01, when extracting DNA from the Bt horizon of the BGL soil type, to 1.64, when extracting DNA from the Of horizon of the TF soil type with method 13.

The DNA yield, calculated using the A_{260} readings, varied considerably with the soil type and the method used. It ranged from 89.5 μg DNA g^{-1} soil for the Ae horizon of the EEB soil using method 1 to 10400 μg DNA g^{-1} soil for the FH horizon of the GEBC soil using method 4 (Table 2.4). On average, methods 4, 5, 13, 12, and 1 yielded 2620, 1800, 1800, 1790, and 1440 μg DNA g^{-1} soil respectively. Method 13 gave the highest average of log(DNA yield) followed by method 12, 4, 5, and 1 (Fig. 2.3)

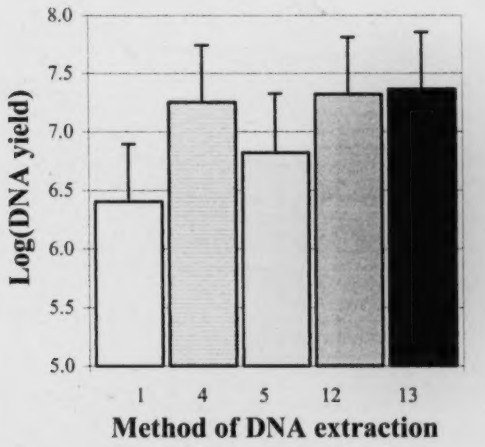

Fig. 2.3 Averages of the log(DNA yield)[a] for each method of direct DNA extraction (the values were not significantly different). [a]DNA yield = μg DNA g^{-1} soil.

However, the MIXED procedure showed that the method of DNA extraction did not have a significant effect on the log(DNA yield) (Table 2.3). The averages for the log(DNA yield) for each combination of method of DNA extraction and soil horizon are presented in Table 2.4. The values of the log(DNA yield) ranged from 4.494, when extracting DNA from the Ae horizon of the EEB soil type with method 1, to 9.250, when extracting DNA from the FH horizon of the GEBC soil type with method 13.

Results of DNA amplification from various soil types using method 12 are presented in Fig. 2.4.

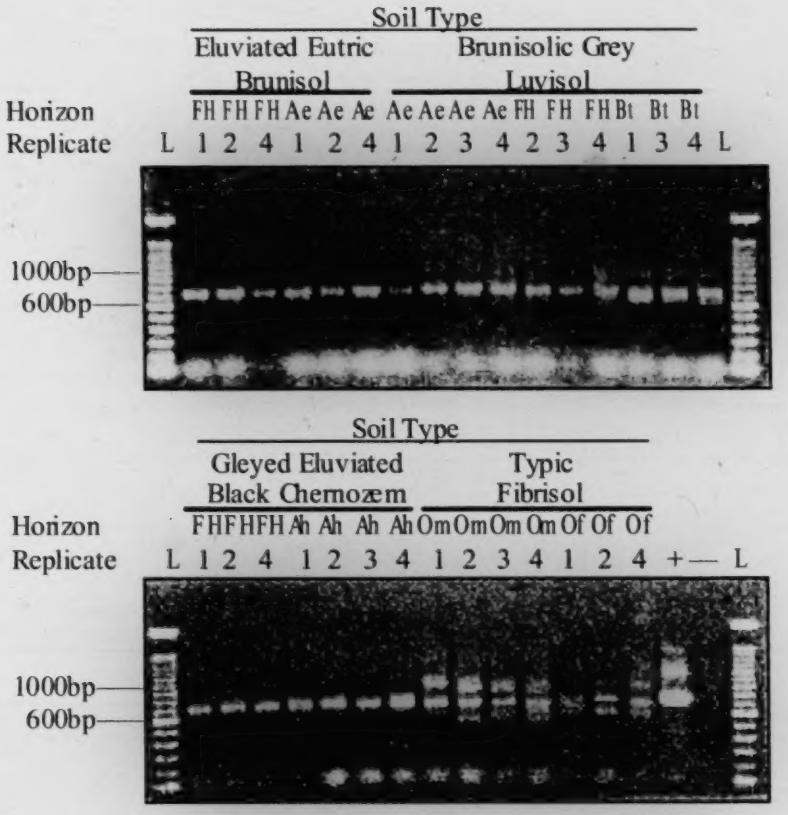
Soil Type
Eluviated Eutric Brunisol
Brunisolic Grey Luvisol
Horizon
FH FH FH Ae Ae Ae Ae Ae Ae Ae Ae FH FH FH Bt Bt Bt
Replicate
L 1 2 4 1 2 4 1 2 3 4 2 3 4 1 3 4 L
1000bp
600bp
Soil Type
Gleyed Eluviated Black Chernozem
Typic Fibrisol
Horizon
F H F H F H Ah Ah Ah Ah Om Om Om Om Of Of Of
Replicate
L 1 2 4 1 2 3 4 1 2 3 4 1 2 4 +— L
1000bp
600bp

Fig. 2.4 PCR products (10µl) obtained from the amplification of DNA extracted with method 12 from four soil types using primers ITS1-F and ITS4. L: 5 µl of 100 bp size ladder; +: 10 µl of PCR amplification with 1/300 of *Laccaria bicolor* DNA; – : PCR negative control (no DNA added). Dilutions used for PCR amplification were: EEB: FH-1-2-4 = 1/450; EEB: Ae-1-2 = 1/150; EEB: Ae-4 = 1/450; BGL: Ae-1 = 1/150; BGL: Ae-2-3-4, FH-2-3-4, Bt-1-3-4: = 1/450; GEBC: FH-1 = 1/150; GEBC: FH-2-4 = 1/450; GEBC: Ah-1= 1/150; GEBC: Ah-2-3-4 = 1/450; TF: Om-1-2-3-4 = 1/450; TF: Of-1-2-4 = 1/150.

All soil types yielded the expected products of about 700 – 800 bp. The BGL and TF soil types also yielded other PCR products, presumably non-specific PCR products or PCR products resulting from the amplification of DNA from plants or other soil organisms. The DNA amplified from *Laccaria bicolor* yielded an expected PCR product of about 750 bp, as well as non-specific products of larger size. The negative control did not give

any amplification, thus the PCR reagents did not contain any DNA that could be amplified with the primers used.

The PCR amplification intensity was evaluated by comparing the intensity of the bands on agarose gel (ranking between 0 and 3). When the PCR amplification is classified as success (nPCR = 1 when PCR = 1, 2, or 3) or failure (nPCR = 0 when PCR = 0), method 12 gave DNA amplification with 100% of the samples followed by method 13 (98%), method 1 (64%), method 5 (9%) and finally method 4, which yielded amplification with only 7% of the DNA samples (Table 2.5).

Table 2.5 Percentages of PCR amplification success for each method of DNA extraction when nPCR = 0 or 1[a].

	% of PCR success	
Method	0	1
1	36	64
4	93	7
5	91	9
12	0	100
13	2	98

[a]nPCR = 0 when PCR = 0
nPCR = 1 when PCR = 1, 2, or 3

The analysis of variance for the PCR amplification success showed that the main factor "method of DNA extraction" had a significant impact on the amplification success at the 95% confidence level (Table 2.3). The averages of the PCR success when PCR = 0, 1, 2, or 3 are presented in Fig. 2.5.

When comparing the intensity of the PCR amplification, there was a significant difference of the results obtained between method 12 and all other methods at the 95% confidence level, method 12 having the highest average. On the other hand, the results obtained with methods 4 and 5 were significantly lower than with the three other methods. There was no significant difference between the averages of the PCR intensity

with methods 1 and 13. The averages of PCR amplification for every combination of method of DNA extraction and soil horizon are presented in Table 2.4.

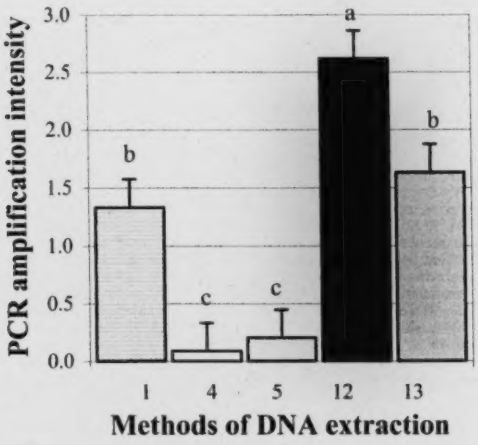

Fig. 2.5 Averages of PCR amplification intensities (between 0 and 3) of DNA extracted with the five methods of DNA extraction tested. 0 = no amplification; 1 = weak amplification; 2 = good amplification; 3 = excellent amplification. Values with no letters in common are significantly different ($\alpha = 0.05$) (Least Square Means).

When ranking the PCR success from 0 to 3, method 12 appeared clearly to give stronger amplification (PCR = 3 in 67% of the samples) more often than the other methods, including method 13 (33% for method 1, 11% for method 13 and 0% for methods 4 and 5) (Table 2.6).

Table 2.6 Percentages of PCR amplification success for each method when PCR = 0, 1, 2, or 3.

	% of PCR amplification success			
Method	0	1	2	3
1	36	24	7	33
4	93	5	2	0
5	91	9	0	0
12	0	7	26	67
13	2	42	45	11

A multiple regression analysis was performed on the A_{260}/A_{230} and the A_{260}/A_{280} ratios, the DNA yield (log[DNA yield]), and the PCR amplification success using the REG procedure and the stepwise selection with $\alpha = 0.15$. Before the regression analysis, an analysis of variance, using the GLM procedure, was done to determine if there was a significant interaction between the main effect "method of DNA extraction" and some of the soil characteristics.

The results of the ANOVA, using the PROC GLM procedure, for the dependent variable A_{260}/A_{230} ratio (using the average of the five samples of each soil horizon), are presented in Table 2.7 and Table 2.8.

Table 2.7 Results of the effect of the model (including Method of DNA extraction, soil characteristics, and Method by soil characteristic interactions) of the ANOVA for the dependent variable A_{260}/A_{230} ratio using the GLM procedure and $\alpha = 0.05$.

Source	DF	Sum of Squares	Mean Square	F-value	Pr> F
Model	49	0.20848	0.00425	4.34	< 0.0001
Error	30	0.02942	0.00098		
Corrected Total	79	0.23790			

The model was significant at $\alpha = 0.05$ (Table 2.7). There were interactions between "method of DNA extraction" and the percentage of organic carbon, of humin, and of HA-B (Table 2.8), therefore, the regression analysis was done for each method separately (Table 2.9).

The first independent variable to enter the model for the humic compound contamination ratio (A_{260}/A_{230}) for method 1 was the percentage of HA-A, which explained 89.84% of the variation of the ratio. The second and final independent variable to enter the model was the percentage of humin which explained 1.86% of the total variation, resulting in the final equation $A_{260}/A_{230} = 0.82616 - 0.00545$ (% HA-A) + 0.00127 (% humin). Thus, these two variables explained 91.70% of the total variation of the A_{260}/A_{230} ratio.

Table 2.8 Results of the ANOVA of the effect of the method of DNA extraction, the soil characteristics, and the interactions between the method of DNA extraction and the soil characteristics for the dependent variable A_{260}/A_{230} ratio using the GLM procedure and $\alpha = 0.05$.

Source	DF	Type III SS	Mean Square	F-value	Pr > F
Method	4	0.00204	0.00051	0.52	0.7211
pH[a]	1	0.00035	0.00035	0.36	0.5532
% of clay	1	0.00547	0.00547	5.57	0.0249
% of silt	1	0.00547	0.00547	5.57	0.0249
% of sand	1	0.00547	0.00547	5.57	0.0249
% of total nitrogen	1	0.00176	0.00176	1.80	0.1902
% of organic carbon	1	0.00004	0.00004	0.04	0.8459
% of humin	1	0.00029	0.00029	0.29	0.5930
% of HA-A	1	0.00019	0.00019	0.20	0.6610
% of HA-B	1	0.00013	0.00013	0.13	0.7198
pH*Method	4	0.00534	0.00134	1.36	0.2704
% of clay*Method	4	0.00204	0.00051	0.52	0.7213
% of silt*Method	4	0.00204	0.00051	0.52	0.7214
% of sand*Method	4	0.00204	0.00051	0.52	0.7213
% of total nitrogen*Method	4	0.00337	0.00084	0.86	0.4993
% of organic carbon*Method	4	0.02538	0.00635	6.47	0.0007
% of humin*Method	4	0.02825	0.00706	7.20	0.0003
% of HA-A*Method	4	0.00315	0.00079	0.80	0.5327
% of HA-B*Method	4	0.02155	0.00539	5.49	0.0019

[a] Measured in a solution of 10 mM $CaCl_2$

Table 2.9 Results of the multiple regression analysis between the dependent variable A_{260}/A_{230} ratio and the soil characteristics for each method of DNA extraction using the REG procedure and $\alpha = 0.15$.

Method	Independent variable	Parameter estimate	F-value	Pr > F	Partial R-Square[a]	Model R-Square
1	Intercept	0.82616	173.58	< 0.0001	ND	ND
	% of HA-A	-0.00545	20.57	0.0005	0.8984	0.8984
	% of humin	0.00127	3.13	0.0984	0.0186	0.9170
4	Intercept	0.81944	9850.86	< 0.0001	ND	ND
	% of HA-A	-0.00436	18.05	0.0009	0.3654	0.3654
	% of organic carbon	0.00147	7.21	0.0187	0.2263	0.5917
5	Intercept	0.79351	357.39	< 0.0001	ND	ND
	% of sand	0.00142	7.42	0.0214	0.4524	0.4524
	% of HA-B	-0.00917	2.49	0.1453	0.1093	0.5617
12	Intercept	0.85099	2358.88	< 0.0001	ND	ND
	% of total nitrogen	-0.03369	3.07	0.1002	0.1698	0.1698
13	Intercept	0.86524	1304.57	< 0.0001	ND	ND
	% of silt	-0.00149	6.99	0.0184	0.3179	0.3179

[a] Partial R-Square values reflect the order in which the variables were placed into the model.
[b]ND: not determined

The percentage of HA-A was also the first variable to enter the model for method 4, with 36.54% of explanation. The second variable to enter the model was the percentage of organic carbon, which explained 22.63% of the variation of the A_{260}/A_{230} ratio, for a final equation of A_{260}/A_{230} = 0.81944 – 0.00436 (% HA-A) + 0.00147 (% organic carbon). Thus, 59.17% of the total variation of the ratio was explained by these two variables.

With method 5, the first variable to enter the model of the A_{260}/A_{230} ratio was the percentage of sand, which explained 42.24% of variation. The last variable left in the model was the percentage of HA-B, which explained 10.93% of the variation, for a total of 56.17% of explanation of the ratio. The final model was A_{260}/A_{230} = 0.79351 + 00142 (% sand) – 0.00917 (% HA-B).

The only variable that entered the model for the humic compound indication ratio with method 12 was the percentage of total nitrogen, which explained 16.98% of the variation, resulting in the final equation A_{260}/A_{230} = 0.85099 – 0.03369 (% total nitrogen).

Finally, the only independent variable that was included in the model for the A_{260}/A_{230} ratio for method 13 was the percentage of silt, which explained 31.79% of the variation. The final equation was A_{260}/A_{230} = 0.86524 – 0.00149 (% of silt).

The results of the ANOVA, using the GLM procedure, for the dependent variable A_{260}/A_{280} ratio (using the average of the five samples of each horizon) are presented in Table 2.10 and Table 2.11. The model was not significant at $\alpha = 0.05$ (Table 2.10).

Table 2.10 Results of the effect of the model (including Method of DNA extraction, soil characteristics, and Method by soil characteristic interactions) of the ANOVA for the dependent variable A_{260}/A_{280} ratio using the GLM procedure and $\alpha = 0.05$.

Source	DF	Sum of Squares	Mean Square	F-value	Pr> F
Model	49	0.84689	0.01728	1.25	0.2536
Error	31	0.42707	0.01378		
Corrected Total	80	1.273964			

Table 2.11 Results of the ANOVA of the effect of the method of DNA extraction, the soil characteristics, and the interactions between the method of DNA extraction and the soil characteristics for the dependent variable A_{260}/A_{280} ratio using the GLM procedure and $\alpha = 0.05$.

Source	DF	Type III SS	Mean Square	F-value	Pr > F
Method	4	0.02745	0.00686	0.50	0.7372
pH[a]	1	0.05509	0.05509	4.00	0.0544
% of clay	1	0.02511	0.02511	1.82	0.1867
% of silt	1	0.02513	0.02513	1.82	0.1866
% of sand	1	0.02514	0.02514	1.82	0.1865
% of total nitrogen	1	0.01958	0.01958	1.42	0.2422
% of organic carbon	1	0.00950	0.00950	0.69	0.4127
% of humin	1	0.00001	0.00001	<0.001	0.9825
% of HA-A	1	0.05002	0.05002	3.63	0.0660
% of HA-B	1	0.03103	0.03103	2.25	0.1435
pH*Method	4	0.01859	0.00465	0.34	0.8507
% of clay*Method	4	0.02742	0.00686	0.50	0.7375
% of silt*Method	4	0.02743	0.00686	0.50	0.7375
% of sand*Method	4	0.02743	0.00686	0.50	0.7375
% of total nitrogen*Method	4	0.00782	0.00195	0.14	0.9653
% of organic carbon*Method	4	0.01676	0.00419	0.30	0.8730
% of humin*Method	4	0.00512	0.00128	0.09	0.9840
% of HA-A*Method	4	0.01240	0.00310	0.23	0.9223
% of HA-B*Method	4	0.00731	0.00183	0.13	0.9692

[a] Measured in a solution of 10 mM $CaCl_2$

There was no significant interaction between "method of DNA extraction" and the soil characteristics, therefore, the regression analysis was done averaging over all methods. No variable met the 0.15 significance level for entry into the model and, therefore, none of the independent variables measured and reported here were correlated with the A_{260}/A_{280} ratio. The results of the regression analysis, when done for each method separately, are presented in Table 2.12.

In this case, the percentage of organic carbon was negatively correlated with the A_{260}/A_{280} ratio and explained 28.36% of the variation when using method 4. When using method 12, the percentage of HA-A was negatively correlated with the A_{260}/A_{280} ratio and explained 16.01% of the variation. Moreover, The pH was positively correlated and explained another 12.29% of the variation of the A_{260}/A_{280} ratio.

Table 2.12 Results of the multiple regression analysis between the dependent variable A_{260}/A_{280} ratio and the soil characteristics for each methods of DNA extraction using the REG procedure and $\alpha = 0.15$.

Method	Independent variable	Parameter estimate	F-value	Pr > F	Partial R-Square	Model R-Square
1	None	ND[b]	ND	ND	ND	ND
4	Intercept	1.24749	1819.20	< 0.0001	ND	ND
	% of organic carbon	-0.00307	5.94	0.0277	0.2836	0.2836
5	None	ND	ND	ND	ND	ND
12	Intercept	1.12374	57.87	< 0.0001	ND	ND
	% of HA-A	-0.00701	5.39	0.0358	0.1601	0.1601
	pH[a]	0.04985	2.40	0.1436	0.1229	0.2830
13	None	ND	ND	ND	ND	ND

[a] Measured in a solution of 10 mM $CaCl_2$
[b]ND: not determined

The results of the ANOVA, using the GLM procedure, for the dependent variable log(DNA yield) (using the average of the five samples of each horizon) are presented in Table 2.13 and Table 2.14. The model was significant at $\alpha = 0.05$ (Table 2.13).

Table 2.13 Results of the effect of the model (including Method of DNA extraction, soil characteristics, and Method by soil characteristic interactions) of the ANOVA for the dependent variable log(DNA yield)[a] using the GLM procedure and $\alpha = 0.05$.

Source	DF	Sum of Squares	Mean Square	F-value	Pr> F
Model	49	97.9150697	1.9982667	7.47	< 0.0001
Error	31	8.2967612	0.2676375		
Corrected Total	80	106.2118309			

[a] DNA yield = μg DNA g^{-1} soil

There was a significant interaction between "method of DNA extraction" and the percentage of HA-B, therefore, the regression analysis was done for each method separately (Table 2.15).

Table 2.14 Results of the ANOVA of the effect of the method of DNA extraction, the soil characteristics, and the interactions between the method of DNA extraction and the soil characteristics for the dependent variable log(DNA yield)[a] using the GLM procedure and $\alpha = 0.05$.

Source	DF	Type III SS	Mean Square	F-value	Pr > F
Method	4	2.58273	0.64568	2.41	0.0710
pH[b]	1	0.76735	0.76735	2.87	0.1004
% of clay	1	1.19196	1.19195	4.45	0.0430
% of silt	1	1.19205	1.19205	4.45	0.0430
% of sand	1	0.19228	1.19228	4.45	0.0430
% of total nitrogen	1	0.15352	0.15353	0.57	0.4545
% of organic carbon	1	0.47521	0.47521	1.78	0.1924
% of humin	1	0.10371	0.10371	0.39	0.5382
% of HA-A	1	0.84483	0.84483	3.16	0.0854
% of HA-B	1	0.00032	0.00032	<0.001	0.9727
pH*Method	4	1.91058	0.47764	1.78	0.1571
% of clay*Method	4	2.58413	0.64603	2.41	0.0700
% of silt*Method	4	2.58214	0.64553	2.41	0.0702
% of sand*Method	4	2.58249	0.64562	2.41	0.0701
% of total nitrogen*Method	4	0.29797	0.07449	0.28	0.8897
% of organic carbon*Method	4	2.73933	0.68283	2.56	0.0820
% of humin*Method	4	0.29863	0.07466	0.28	0.8893
% of HA-A*Method	4	0.47187	0.11797	0.44	0.7781
% of HA-B*Method	4	2.98839	0.74710	2.79	0.0434

[a] DNA yield = μg DNA g^{-1} soil
[b] Measured in a solution of 10 mM $CaCl_2$

The models for the log(DNA yield) were more complex than for the A_{260}/A_{230} ratio. The first variable entered in the model for method 1 was the percentage of HA-A, which was positively correlated and explained 60.87% of the variation of the log(DNA yield), followed by the percentage of silt, which explained 14.23% of the variation. The third variable to enter the model was the percentage of HA-B, which was also positively correlated and explained 4.37% of the variation. These three variables explained 79.47% of the total variation of the log(DNA yield) for a final equation of log(DNA yield) = 3.8319 + 0.08562 (% HA-A) + 0.03164 (% silt) + 0.26445 (% HA-B).

Table 2.15 Results of the multiple regression analysis between the dependent variable log(DNA yield)[a] and the soil characteristics for each method of DNA extraction using the REG procedure and $\alpha = 0.15$.

Method	Independent variable	Parameter estimate	F-value	Pr > F	Partial R-Square	Model R-Square
1	Intercept	3.83190	61.32	< 0.0001	ND[b]	ND
	% of HA-A	0.08562	3.57	0.0814	0.6087	0.6087
	% of silt	0.03164	11.59	0.0047	0.1423	0.7510
	% of HA-B	0.26445	2.77	0.1201	0.0437	0.7947
4	Intercept	9.60969	157.91	< 0.0001	ND	ND
	% of HA-A	0.22877	172.52	< 0.0001	0.8105	0.8105
	pH	-1.03825	27.97	0.0001	0.0924	0.9029
	% of sand	0.01458	8.05	0.014	0.0371	0.9400
5	Intercept	4.76066	255.34	< 0.0001	ND	ND
	% of HA-A	0.19229	27.95	0.0005	0.8024	0.8024
	% of organic carbon	0.05896	11.19	0.0086	0.0695	0.8719
	% of total nitrogen	-1.40595	4.15	0.0722	0.0404	0.9123
12	Intercept	8.26072	191.63	< 0.0001	ND	ND
	% of organic carbon	0.03866	37.62	< 0.0001	0.5769	0.5769
	pH	-0.35791	7.85	0.0141	0.152	0.7289
13	Intercept	8.28163	138.02	< 0.0001	ND	ND
	% of organic carbon	0.02258	9.20	0.0089	0.2506	0.2506
	pH	-0.29097	3.72	0.0744	0.1572	0.4079

[a] DNA yield = $\mu g\ DNA\ g^{-1}$ soil
[b] ND: not determined

The percentage of HA-A was also the first variable to be incorporated into the model of method 4 for the log(DNA yield), and explained 81.05% of the variation of this dependent variable. The pH, which was negatively correlated, added another 9.24% of the variation, followed by the percentage of sand, which explained 3.71% of the variation, for a total of 94.00% of explanation of the variation of the log(DNA yield). The final equation was log(DNA yield) = 9.60969 + 0.22877 (% HA-A) – 1.03825 (pH) + 0.01458 (% sand).

When using method 5, the percentage of HA-A was also the first independent variable to enter the model and explained 80.24% of the variation of the log(DNA yield). Another 6.95% of the variation was explained by the percentage of organic carbon. The last soil characteristic to enter the model was the percentage of total nitrogen, which explained 4.04% of the variation. Thus, these three variables explained a total of 91.23% of the

variation of the log(DNA yield). The final equation was log(DNA yield) = 4.76066 + 0.19229 (% HA-A) + 0.05896 (% organic carbon) – 1.40595 (% total nitrogen).

When DNA was extracted and purified using method 12, the variation in the log(DNA yield) was positively correlated with the percentage of organic carbon, which explained 57.69% of the variation, and negatively correlated with the pH, which explained 1.41% of the variation. Thus, a total of 72.89% of the variation of the log(DNA yield) was explained by these two variables, resulting in the final model log(DNA yield) = 8.26072 + 0.03866 (% organic carbon) – 0.35791 (pH).

When using method 13, the same two variables entered the model, with the percentage of organic carbon explaining 25.06% of the total variation of the log(DNA yield), and the pH explaining 15.72%, for a total of 40.79%. With this method, the final model was log(DNA yield) = 8.28163 + 0.02258 (% organic carbon) – 0.29097 (pH).

Because the Pr > F was close to the α value (Pr > F = 0.0434), the results of the regression analysis are also presented when averaging over all methods in Table 2.16.

Table 2.16 Results of the multiple regression analysis between the dependent variable log(DNA yield)[a] and the soil characteristics averaging over all methods of DNA extraction using the REG procedure and α = 0.15.

Independent variable	Parameter estimate	F-value	Pr > F	Partial R-Square	Model R-Square
Intercept	8.00001	123.82	< 0.0001	ND[b]	ND
% of HA-A	0.07497	7.01	0.0099	0.4525	0.4525
pH	-0.33726	3.04	0.0852	0.0720	0.5245
% of organic carbon	0.02735	5.38	0.023	0.0185	0.5431
% of sand	-0.00902	2.23	0.1385	0.0130	0.5561

[a] DNA yield = μg DNA g^{-1} soil
[b] ND: not determined

When overaging over all methods of DNA extraction, the percentage of HA-A was positively correlated with the log(DNA yield) and explained 45.25% of the variation. The

pH was negatively correlated with the log(DNA yield) and was the second variable entered in the model, with 7.2% of explanation. The percentage of organic carbon was positively correlated and explained 1.85% of the total variation, followed by the percentage of sand, which was negatively correlated and explained an additional 1.3% of the variation of the log(DNA yield).

The results of the ANOVA, using the GLM procedure, for the dependent variable PCR amplification (using the average of the five samples of each soil horizon), are presented in Table 2.17 and Table 2.18. The model was significant at $\alpha = 0.05$ (Table 2.17).

Table 2.17 Results of the effect of the model (including Method of DNA extraction, soil characteristics, and Method by soil characteristic interactions) of the ANOVA for the dependent variable PCR amplification (PCR = 0, 1, 2, or 3) using the GLM procedure and $\alpha = 0.05$.

Source	DF	Sum of Squares	Mean Square	F-value	Pr> F
Model	49	104.14576	2.12542	9.38	< 0.0001
Error	31	7.02159	0.22650		
Corrected Total	80	111.16735			

There was no interaction between "method of DNA extraction" and the soil characteristics, so the regression analysis was done on the average values of the PCR amplification for all methods together (Table 2.19).

The only variable to enter the model of the PCR amplification success was the percentage of total nitrogen, which explained only 8.85% of the results of the PCR amplification, for a final equation of PCR = 1.72256 – 0.66275 (% total nitrogen). Thus, the higher the percentage of total nitrogen in the soil prior to DNA extraction, the smaller the chances are to amplify DNA with PCR. However, it explained only 8.85% of the results of PCR amplification, even if it was significant at the 85% confidence level.

Table 2.18 Results of the ANOVA of the effect of the method of DNA extraction, the soil characteristics, and the interactions between the method of DNA extraction and the soil characteristics for the dependent variable PCR amplification (PCR = 0, 1, 2, or 3) using the GLM procedure and $\alpha = 0.05$.

Source	DF	Type III SS	Mean Square	F-value	Pr > F
Method	4	0.73778	0.18444	0.81	0.5258
pH[a]	1	0.85660	0.85660	3.78	0.0609
% of clay	1	0.41889	0.41889	1.85	0.1837
% of silt	1	0.41667	0.41667	1.84	0.1848
% of sand	1	0.41696	0.41696	1.84	0.1846
% of total nitrogen	1	0.46024	0.46024	2.03	0.1640
% of organic carbon	1	0.06590	0.06590	0.29	0.5935
% of humin	1	0.66255	0.66255	2.93	0.0972
% of HA-A	1	0.25060	0.25060	1.11	0.3010
% of HA-B	1	0.00002	0.00002	0.00	0.9923
pH*Method	4	0.34358	0.08589	0.38	0.8217
% of clay*Method	4	0.73831	0.18458	0.81	0.5254
% of silt*Method	4	0.73767	0.18442	0.81	0.5259
% of sand*Method	4	0.73780	0.18445	0.81	0.5258
% of total nitrogen*Method	4	0.83130	0.20783	0.92	0.4662
% of organic carbon*Method	4	0.88009	0.22002	0.97	0.4372
% of humin*Method	4	1.31014	0.32754	1.45	0.2424
% of HA-A*Method	4	0.66245	0.16561	0.73	0.5776
% of HA-B*Method	4	0.92022	0.23005	1.02	0.4145

[a] Measured in a solution of 10 mM $CaCl_2$

Table 2.19 Results of the multiple regression analysis between the dependent variable PCR amplification (PCR = 0, 1, 2, or 3) and the soil characteristics averaging over all methods of DNA extraction using the REG procedure and $\alpha = 0.15$.

Independent variable	Parameter estimate	F-value	Pr > F	Partial R-Square	Model R-Square
Intercept	1.72256	59.55	< 0.0001	ND[b]	ND
% of total nitrogen	-0.66275	7.67	0.007	0.0885	0.0885

[b] ND: not determined

2.5. Discussion

The objective of this study was to develop a method that would work with all the soil types tested. When a method was tried with only one or two soil types, it does not necessarily mean that this method would not have given better results with the other soil types.

When looking at the A_{260}/A_{230} ratios, if humic substances were the major factor inhibiting PCR amplification, method 1 would have been expected to give the highest amplification success, because the DNA purified with this method had higher ratio values. However, method 12, which produced DNA extracts with smaller A_{260}/A_{230} ratios, i.e. supposedly more contaminated with humic compounds, led to the highest rate of PCR amplification. Nevertheless, the multiple regression analysis showed that the A_{260}/A_{230} ratio was negatively correlated with the percentage of HA-A when the DNA was extracted with methods 1 and 4, meaning that this ratio was still relevant to at least a certain point. With method 5, the percentage of HA-B was negatively correlated with the A_{260}/A_{230} ratio and explained 10.93% of the variation of the values. Thus, the results obtained with the regression analysis suggested that methods 1, 4 and 5 left a fair amount of humic compounds in the DNA extracts.

The second variable to enter the model when using method 1 was the percentage of humin, which was positively correlated to the A_{260}/A_{230} ratio but explained only 1.86% of the variation. With method 4, the percentage of organic carbon was positively correlated and explained 22.63% of the total variation. Other molecules could have absorbed at 260 nm, and if they were not also absorbing at 230 nm (or inversely), the ratios obtained might not be accurate estimates of the humic substance contamination.

The percentage of sand was the first independent variable to enter into the model with method 5. It was positively correlated with the A_{260}/A_{230} ratio and explained 45.24% of the variation. When the amount of sand increases in a soil sample, chances are that the amount of other compounds, including humic acid compounds, will decrease, and thus, higher A_{260}/A_{230} ratios will be obtained, because DNA is easily recovered from sands when the sample is centrifuged.

Neither HA-A nor HA-B was part of the model for the humic acid indicator ratio when using methods 12 and 13. Thus, they probably were more efficient in removing humic compounds from the DNA extracts. This is suported by the fact that the DNA extracts obtained with methods 12 and 13 were usually colorless. The only variable that entered

the model with method 12 was the percentage of total nitrogen, which explained 16.98% of the variation of the ratio. Thus, the increase of compounds containing nitrogen left in the extract decreased the A_{260}/A_{230} ratio. With method 13, it was the increase in the percentage of silt that was negatively correlated with the same ratio, and explained 31.79% of the variation of the ratio. If some variables were correlated with each other, it might have hidden their impact on the A_{260}/A_{230} ratio.

No soil characteristics met the 85% confidence level to enter into the model of the A_{260}/A_{280} ratio, and thus, none explained its variation. Since proteins have a significant amount of nitrogen, and the A_{260}/A_{280} ratios are supposed to reflect the amount of protein contamination in the DNA extracts, the percentage of total nitrogen was expected to have a great influence on this ratio. However, other molecules could have absorbed at 260 nm and not at 280 nm (or inversely), and thus, the ratios obtained might not be accurate estimates of the protein contamination.

The accuracy of the DNA yields calculated using the A_{260} readings can be questioned considering the enormous values obtained (from 89.5 to 10400 μg DNA g^{-1} soil) compared to the values reported by other authors, regardless of the method or soil type used. For example, the highest yield obtained by Frostegård *et al.* (1999) was 160 ± 7 μg DNA g^{-1} sandy clay loam soil. All other papers reviewed reported lower yields of DNA, even for forest soils (Zhou *et al.*, 1996; Miller *et al.*, 1999). It is possible that other molecules present in the DNA extracts absorbed at the same wavelength as DNA, thus, increasing the A_{260} and resulting in inaccurately high estimates of the amounts of DNA. Steffan *et al.* (1988) have previously mentioned that without extensive purification, the A_{260} determinations were not true measures of the amount of DNA. Indeed, Cullen and Hirsch (1998) have demonstrated that spectrophotometric A_{260} measurements lack specificity for DNA due to interference from proteins, RNA, nucleotides, some detergents, and other contaminants, which also absorb at 260 nm, and that co-extracted humic substances can lead to an overestimate of the DNA content by up to ten-fold. This fact can be depicted from the analysis of the impact of the independent variables on the log(DNA yield), since the first variable to enter the model for methods 1, 4, and 5 was the

HA-A content, which was positively correlated and explained 60.87%, 81.05%, and 80.24% of the variation of the log(DNA yield) calculated, respectively. Therefore, these results suggested that these three methods did not efficiently remove humic compounds from DNA. For more accurate quantification of DNA, Cullen and Hirsch (1998) mentioned that fluorometry using Hoechst 33258 (bis-benzimidazole) (Sigma, U.K.) is specific for double-stranded DNA because the readings are not subject to interference by the above components. However, high amounts of humic material still have an effect on the readings.

The reason why the percentage of HA-A was not included in the model of log(DNA yield) for methods 12 and 13 might be that these two methods were more efficient in removing humic compounds in the DNA extract, and thus did not significantly affect the readings of A_{260}. When using methods 12 and 13 to purify DNA, the percentage of organic carbon was positively correlated with the log(DNA yield), and explained 57.69% and 25.06% of the variation, respectively. The percentage of organic carbon was also in the model of the log (DNA yield) for method 5, but explained only 6.95% of the total variation. One can predict that the presence of organic carbon could indicate the presence of cells from diverse organisms, dead or alive, which would all contain DNA. It is important to remember that the percentage of organic matter was calculated using the percentage of organic carbon, so the percentage of organic matter would also be expected to be correlated with the log(DNA yield). Moreover, HA-A is a fraction of organic matter and is composed of organic carbon, so its inclusion in some of the models could explain the small variation brought about by the percentage of organic carbon. Zhou *et al.* (1996) also found that the soil organic carbon content was positively correlated ($r = 0.73$) with the DNA yields obtained in their experiment. The reason the percentage of HA-B was only included in the model for method 1, where it only explained 4.37% of the variation of the log(DNA yield), is that HA-B is the fraction of humic acids that is bound to clay particles. Thus, it was expected to be eliminated early in the DNA purification, when the samples were centrifuged the first time.

The pH, which was negatively correlated to the same variable, was the second independent variable included in the model of the log(DNA yield) for methods 4, 12, and 13 and explained 9.24%, 15.20%, and 15.72% of the variation, respectively. Therefore, the log(DNA yield) is expected to increase with a decrease in the pH, but the reason remains unknown.

The second variable to enter the model with method 1 was the percentage silt, which was positively correlated with the log(DNA yield) and explained an additional 14.23% of the variation. With method 4, the percentage of sand was positively correlated but explained only 3.71% of the variation of the log(DNA yield). Frostegård *et al.* (1999) have reported that clay content strongly negatively influenced the recovery of DNA from soil, probably because of DNA adsorption to clay particles. However, only sand and silt content were positively correlated with the log(DNA yield) when using methods 1 and 4 in the present study.

Cell lysis efficiency is another factor that influences the amount of DNA recovered from soil samples, and Zhou *et al.* (1996) observed that it was significantly correlated with the clay content of the soils ($r = -0.67$). However, no attempt was made to evaluate the cell lysis efficiency in the present study.

Finally, when using method 5, the last variable to enter the model of the log(DNA yield) was the percentage of total nitrogen, which was negatively correlated at the 85% confidence level but only explained 4.04% of the variation of the log(DNA yield). Again, if some soil characteristics were correlated with each other, it might have hidden their impact on the log(DNA yield).

Tsai and Olson (1992b) have tested the inhibitory effect of pure humic acid (Aldrich) on PCR and have found that, with the conditions they used, concentrations as low as 10 ng could suppress the PCR. When using humic-acid-like substances obtained from sediments, an amount of 27 μg was sufficient to inhibit the PCR amplification. As previously mentioned, humic substances can bind to DNA, preventing *Taq* polymerase

(and primers) from binding to it and synthesizing the complementary DNA strand. In the present study, the effect of pure humic acids or humic acids extracted from soil samples was not tested, but the soil HA-A and HA-B contents were not found to be correlated with the PCR amplification success. Because methods 12 and 13 yielded very clear DNA extracts, the amount of humic substances was probably very low and was, therefore, not expected to influence the PCR amplification success. Instead, the percentage of total nitrogen was negatively correlated with the PCR amplification success (results when averaging over all methods together), but explained only 8.85% of the intensity of amplification (between 0 and 3). Young *et al.* (1993) found that when using gel purification, there was an inverse correlation between the yield of DNA amplification and the organic content of the soil from which the DNA was isolated, but results of the gel purification have not been included in the analyses because of missing data. Along with the amount of total nitrogen, other things, such as metal ions and fulvic acid (Tsai and Olson, 1992b), might also have inhibited the PCR reaction. Moreover, many other factors might have played a role in the failure of the DNA amplification. For example, in addition to the inhibitors present in the DNA extracts, presence of non-target DNA in the PCR reaction can competitively inhibit *Taq* polymerase in the DNA synthesis reaction (Tebbe and Vahjen, 1993; Cullen and Hirsch, 1998).

Considering that the ratios for the purity (A_{260}/A_{230} and A_{260}/A_{280} ratios) of DNA and the calculation of its concentration in the extracts using the A_{260} may not be completely reliable, and that the ratios for the purity did not necessarily reflect the ability to amplify DNA, the only good criteria for the selection of a DNA purification method were the amplification success of DNA by PCR, the time required to do the purification, and the cost of the material used. However, the cost of the methods was not precisely evaluated in this study.

Methods 12 and 13 not only gave the highest probability of PCR success (100% and 98%, respectively) (Table 2.5) compared to the three other methods analysed, but they also led to successful DNA amplification with all samples (except for the DNA of one sample of the Ah horizon of the GEBC soil extracted with method 13). Thus, these two

methods of direct DNA extraction gave consistent results for the four soil types selected in this study, an important criterion when selecting a method of DNA extraction to use with soil samples. However, method 12 more often gave an amplification of 3 (PCR = 3) compared to method 13 (67% and 11%, respectively), a factor that could be important when the target DNA is in small concentration compared to the non-target DNA present in the extracts. Moreover, although there was no significant difference between the results obtained with methods 12 and 13 for the dependant variables A_{260}/A_{230} and A_{260}/A_{280} ratios, and log(DNA yield), method 12 was somewhat quicker than method 13 because the columns were already filled with the Sephacryl™ HR resin (Sephacryl S-400 HR) and gave significantly higher PCR amplification intensity. Also, with method 13, some DNA extracts needed to pass through more columns than with method 12 to be suitable for PCR amplification. However, smaller aliquots of crude DNA extracts can be loaded in the columns of the two methods to avoid the need to use more than one column for the purification. Frostegård *et al.* (1999) have also used Sephacryl S-400 HR columns but the DNA extraction protocol was not the same and the elution through this type of column was followed by elution through an Elutip-d column (Schleicher & Schuell, Dassel, Germany). Thus, our method was less expensive and somewhat faster.

2.6. Conclusion

The goal of this study was to develop a method of direct DNA extraction that would work with a variety of different soil types. Methods 12 and 13 worked with all the soils tested and they could be tried on other challenging soil types with extreme values of physical and chemical properties to assess their efficiency.

Despite the present limitations, methods 12 and 13 of DNA extraction and purification developed here, which use Sephacryl™ S-400 HR columns, and PVPP columns, respectively, yielded DNA suitable for PCR amplification from all the soil types tested in a short period of time (2-3 h and 2.25-3.50 h to process 10 samples, respectively) and are environmentally friendly, because they use safe reagents compared to other methods.

They will be invaluable in soil microbial and molecular ecology studies, because they are simpler, faster, and very efficient in producing DNA suitable for PCR compared to many existing methods. Although method 13 yielded PCR products with 98% of the samples, method 12 was faster and gave DNA that could be amplified from all 45 samples tested.

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CHAPTER 3

TEST OF A WHOLE SOIL DNA EXTRACTION METHOD FOR PCR AMPLIFICATION OF ENDOMYCORRHIZAL FUNGI FROM TREMBLING ASPEN FORESTS IN NORTHERN ALBERTA AND QUÉBEC

3.1 Introduction

While most plants show strong fidelity to a particular mycorrhiza type, species of *Populus* can form arbuscular mycorrhizae (AM) either solely or simultaneously with ectomycorrhizae, sometimes in the same rootlet (Chilvers *et al.*, 1987; Khasa *et al.*, 2002). Aspen is colonized by both ectomycorrhizal and endomycorrhizal fungi, but predominantly by ectomycorrhizal fungi (Cripps and Miller, 1993). However, the importance of these mycorrhizal associations remains mostly unexplored (Pregitzer and Friend, 1996). Thus, a single tree will form different types of mycorrhizae relationships during different stages of its life cycle, or under different environmental conditions (Lodge and Wentworth, 1990). Mycorrhizal members of the Cortinariaceae (e.g., *Inocybe* species) are an important component of the mycoflora of aspen, which can be host to a large number of ectomycorrhizal fungal species (Cripps and Miller, 1993; Cripps, 1997). As well, aspen is colonized by the glomalean fungi (e.g., *Glomus* species), which form endomycorrhizal symbioses (Glomales, Zygomycotina). However, poplars can develop even without mycorrhizae, which enable the trees to colonize disturbed sites where mycorrhizal fungal inoculums may not be available (Schultz *et al.*, 1983 - as described by Peterson and Peterson, 1992).

On sites with low to moderate soil fertility, mycorrhizal fungi associations are essential to poplar (Dickmann *et al.*, 2001). The symbiotic association between mycorrhizal fungi and roots is especially important for the absorption of immobile nutrients such as P, but mycorrhizae also help to acquire N, K, Ca, S, and Zn. Fungal hyphae release enzymes that enable them to digest and penetrate substrates, assisting the chemical breakdown of

organic substances, which are then used by the fungus and/or its host as nutrients (Laursen, 1985). Without these associations, the uptake of nutrients can be as low as one-sixth of that of infected roots (Smith and Read, 1997).

The hyphae of endomycorrhizae, which actually penetrate into the cells of the roots, are more common on young poplars (Dickmann *et al.*, 2001). On the other hand, ectomycorrhizae penetrate between the cell walls of the root and sometimes completely envelop the tips in a mantle, thereby changing their morphology (Dickmann *et al.*, 2001). The network of mycorrhizal hyphae is much smaller than the finest poplar roots and grows into the soil where it takes up nutrients from minute soil pores that are inaccessible to tree roots (Dickmann *et al.*, 2001).

Mycorrhizal associations, both with ectomycorrhizal and endomycorrhizal fungi, usually form on fine roots of poplars (Dickmann *et al.*, 2001; Khasa *et al.*, 2002). As stands mature and the soil organic matter builds up, infection tends to shift from endo- to ectomycorrhizal (Heilman *et al.*, 1996). Like aspen, AM fungi are important as pioneer colonizers (Forster and Nicolson, 1981). Lodge and Wentworth (1990) demonstrated that *Populus* may form both AM and ectomycorrhizae, but on different root segments, and suggested that the ectomycorrhizae gradually replace the AM. Heavily infected root systems may have as much as 75-100% higher respiration rate compared to non-infected roots (Smith and Read, 1997). This might result in three to four times more carbon allocated to root systems compared to non-mycorrhizal plants (Dickmann *et al.*, 2001). The fungal hyphae of mycorrhizal roots, in return, effectively scavenge a greater volume of soil for water and mineral nutrients than non-mycorrhizal roots, and also can deter invasion by pathogenic fungi (Dickmann *et al.*, 2001).

Mycorrhizal fungi affect plants in several ways. For example, Hooker *et al.*, (1992) has shown that AM fungi induced alterations in poplar root system morphology. In this case, *Scutellospora calospora* (Nicolson & Gerdemann) Walker & Sanders and *Glomus* sp E3 or *Glomus caledonium* (Nicolson & Gerdemann) Trappe and Gerdemann did not affect plant size but induced an increase in lengths of individual secondary and tertiary roots

and an increase in root branching. Rygiewicz and Andersen (1994) have reported a direct measurement of carbon in and through all major pools of a mycorrhizal (fungus-root) coniferous seedling. They noted that mycorrhizal symbionts reduced overall retention of carbon in the plant-fungus symbiosis by reducing its retention and release above ground and by increasing carbon in roots and below-ground respiration. They also noticed that mycorrhizae altered the size of below-ground carbon pools, the quality and, therefore, the retention time of carbon below-ground. Moreover, the mycorrhizal plant shifted allocation of carbon to pools that are rapidly turned over, primarily to fine roots and fungal hyphae, and host root fungal respiration.

In mountain and subalpine forests, many organisms depend upon the varied and unique habitat provided by aspen stands, making this tree an important contributor to biodiversity in those areas (DeByle and Winokur, 1985). It is also useful for reclamation and reforestation efforts because of its ability to rapidly colonize an area by cloning (Cripps and Miller, 1993). With its economical importance, those latest two aspects highlight the need to better manage aspen stands.

Mycorrhizal fungi benefit trees by augmenting inorganic nutrient uptake and providing protection from heavy metals, drought, pathogens, grazers, and other organisms (Fogel, 1980). It is possible that the diversity of fungi below ground in trembling aspen stands enhances the ability of aspen to survive a variety of conditions. It is, therefore, important to have a better picture of the mycorrhizal component in trembling aspen stands in order to improve management practices and yields, as well as to have a better general understanding of the dynamics of its ecosystem.

There are more than 150 species of AM fungi (Morton, 1988), which are ubiquitous in soil and have great potential to enhance plant growth and soil aggregation. AM fungi are endomycorrhizal fungi that form networks of extraradical hyphae, but after the formation of specialized fungal structure, the appressoria, root colonization proceeds with the growth of intraradical hyphae (Bianciotto and Bonfante, 2002). These intraradical hyphae cross the epidermal layer, and eventually the root colonization procceeds with the

formation of intracellular, highly branched structures termed arbuscules, which are exclusively located in cortical cells (Bianciotto and Bonfante, 2002).

These obligate root symbionts colonize over 80% of terrestrial plant species in nature. The interaction between AM fungi and their hosts is not recognized as specific but the formation of a functional mycorrhizal association is a highly regulated process that is accomplished by molecular changes in both partners during the symbiosis. The benefits of the association between these fungi and the host plant are related to the timing and extent of colonization of roots (Abbott and Gazey, 1994). Phosphate concentration is the major environmental factor affecting AM, with high concentrations inhibiting their development.

Many methods have been used to identify mycorrhizal fungi such as visual differences in morphology of fungal hyphae and of vesicules within roots (Abbott, 1982; Lopez-Aiguillon and Mosse, 1987). However, morphology may change with host (Daniels-Hetrick *et al.*, 1985; Boyetchko and Tewari, 1990), between roots of different ages on the same plant (Hepper, 1985), and with changes of edaphic factors (Mosse, 1973; Amijee *et al.*, 1989). Moreover, closely related fungi may have similar morphology. Thus, depending on the level of specificity needed, other techniques of identification are available, such as serological methods including immunological techniques, which may not be suitable because the reactive sites are not always on the surface of the fungal hyphae (Wilson *et al.*, 1983), or molecular techniques like the specific polymerase chain reaction (PCR) amplification. The later technology is becoming more popular because of its specificity and its sensitivity.

The use of PCR and specific gene markers are very helpful to identify organisms and genotypes where morphology does not give enough information, such as for fungi in mycorrhiza (Bachmann, 1994). The fungal DNA must be specifically amplified and the amplified product can be analysed by RFLP (restriction fragment length polymorphisms), sequencing or oligonucleotide probing (Gardes and Bruns, 1993).

Analysis of rRNA (ribosomal RNA) operons is especially useful, because RNA is present in all cells and contains highly conserved regions, as well as variable ones (Woese, 1987 - as described by Hill *et al.*, 2000). The highly conserved regions can be used to design PCR primers, and the variable regions between the conserved ones can be amplified (Erb and Wagner-Döbler, 1993). In the eukaryotic nuclear ribosomal RNA genes, the internal transcribed spacer (ITS) fragment is a variable region that contains two non-coding spacers and is typically present as a tandem array of 100 or more identical or nearly identical copies (Gardes and Bruns, 1996b). The highly conserved sequences of ribosomal 18S, 5.8S and 28S are interrupted by the ITS1 (between 18S and 5.8S) and ITS2 (between 5.8S and 28S). These regions are often very variable among different fungal species but highly conserved within species (Gardes and Bruns, 1993). With the fungal-selective primer ITS1-F, amplification of DNA from all ascomycetes and basidiomycetes tested by Gardes and Bruns (1993) was successful. However, ITS4-B (which is specific to basidiomycetes), when paired with ITS1-F, efficiently amplified DNA from all basidiomycetes, discriminating against ascomycete DNAs but produced a small amount of PCR products from certain plant species. These primers (ITS1-F and ITS4-B) can be useful for the detection and analysis of the basidiomycete component in ectomycorrhizae (Gardes and Bruns, 1993). However, their annealing temperatures differ by about 12°C so the amplification may not be optimal. It is also possible to analyse the highly polymorphic intergenic spacer region (IGS) of the rDNA (Erland *et al.*, 1994; Pritsch *et al.*, 1997). Erland *et al.* (1994) have found that this region is more variable than the ITS region in several fungal species, but the results obtained by Pritsch (1996) (as described by Pritsch *et al.*, 1997) showed that in the case of black alder mycorrhizas, not all of the species could be amplified.

Most of the studies done on mycorrhizal fungi in natural environments using molecular techniques were performed on DNA from sampled fruiting bodies and mycorrhizae (Gardes and Bruns, 1993, 1996b; Pritsch *et al.*, 1997), which require a high number and density of samples to achieve a complete survey over a long period of time. For example, Cripps and Miller (1993) collected sporocarps over three field seasons in aspen stands, and their results suggested that it might need a longer-term study to record all the

ectomycorrhizal species present. Also, Gardes and Bruns (1996b) have demonstrated with molecular studies that there is a considerable lack of correspondence between the above- (fruiting bodies) and below-ground (ectomycorrhizae) communities of ectomycorrhizal colonizers. For example, the presence of fruiting bodies is indicative of the presence of the species in the soil, but absence of fruiting bodies does not mean the absence of the species in the soil.

A better representative picture of the whole community of mycorrhizal fungi can be obtained using total DNA extracted from soil sampled in forest stands More specifically, with AM fungi, isolations of the small subunit rDNA (referred to as the 18S or SSU gene) have been done from colonized roots (Simon *et al.*, 1992, 1993; Clapp *et al.*, 1995; Di Bonito *et al.*, 1995; Helgason *et al.*, 1998), from individual spores (Sanders *et al.*, 1995; Simon, 1996a,b; Bago *et al.*, 1998), or from soil (Claassen *et al.*, 1996; Chelius and Triplett, 1999).

The VANS1 primer, when paired with a suitable downstream primer, is capable of amplifying the ribosomal genes of only the Glomalean fungi (Simon, 1996a,b). Because endomycorrhizal hyphae are finely intercalated with soil particles and may be difficult to physically separate, separation of cells from the soil matrix prior to cell lysis may not be reliable (Claassen *et al.*, 1996). Claassen *et al.* (1996) have developed a method for direct DNA extraction from soil. They amplified endomycorrhizal fungal DNA with PCR using VANS1 and NS4 as primers (Simon *et al.*, 1992), which amplify a 1.10 kb region specific to the nuclear 18S rDNA from vesicular-arbuscular or endomycorrhizae. However, this method was used following greenhouse culture of a single endomycorrhizal fungi (*Glomus clarum*) and, thus, did not represent the abundance and diversity of DNA from different organisms present in the environment. Consequently, the PCR conditions and, therefore, the PCR amplification efficiency did not reflect what would be obtained in natural environments, where a higher degree of competition for the primers and the *Taq* polymerase from DNA of other organisms is present. Moreover, the method was time consuming (more than 24 h) and the soil used was low in organic carbon compared to most of the soil horizon used in the development of the DNA

extraction method presented in Chapter 2. Similarly, the direct DNA extraction method of Chelius and Triplett (1999), although rapid and simple (producing high quality DNA in about 1 h), was using soil from intensively managed turfgrass, which might not be as recalcitrant as the soils used in Chapters 2 and 3 to yield DNA suitable for PCR amplification.

In some cases, sequencing of the PCR products might be a better approach than RFLP for the identification of mycorrhizal fungi. For example, Vrålstad *et al.* (2000) has demonstrated the limitation of PCR-RFLPs as a method of defining fungal 'taxa' in diversity studies since they have found that specific ectomycorrhizal morphotype may comprise several RFLP types. Gardes and Bruns (1993, 1996a,b) have also found that some closely related species may be lumped together by ITS-RFLP match criterion, and more rarely, intraspecific differences may result in failure to recognize a species match.

As molecular techniques improve in efficiency, the use of PCR on DNA extracted from soil field samples will become more and more useful in elucidating the fine community structure of both endomycorrhizal and ectomycorrhizal fungi.

With molecular tools such as PCR amplification, RFLP, and sequencing of DNA, it would, therefore, be possible to study the genetic and functional diversity of mycorrhizal fungi, and use mycorrhizal technology more efficiently in managing ecosystems of aspen and in inoculation programs. However, effectiveness of inoculation may vary with different tree hosts, fungal species and the level of fertility. Usually, mycorrhizal fungi have a greater potential in soils that are deficient in phosphorus for plant growth (Dodd and Thomson, 1994). Two advantages of using mycorrhizal fungi to improve tree growth and survival compared to fertilizers and phytocides are that they are friendly to the environment and are selective (Villeneuve *et al.* 1991). Depending on the specificity of the fungi, most of the competitive understory will not be affected by the inoculum. Also, mycorrhizal fungi increase tolerance to saline conditions and to sites polluted by heavy metals, improve water relations, and control root disease (Dodd and Thomson, 1994).

3.2 Research Objective

The objective of this study was to determine the endomycorrhizal fungal symbiotic community structure and diversity of trembling aspen forests in northern Alberta and Québec using the Sephacryl S-400 HR MicroSpin™ columns (Amersham Pharmacia Biotech Inc.) method of direct DNA extraction described in Chapter 2 (method 12).

3.3 Materials and Methods

3.3.1 Sampling and soil preparation

Soil samples were collected in the summers of 1999 and 2000 from high performing aspen stands using soil core samplers. The performance of the stands was determined using a site index map. Enough soil was sampled to do physical and chemical analyses to allow comparisons to be made between physico-chemical properties and mycorrhizal fungi. The biological samples were frozen at –20°C upon arrival at the University of Alberta or at Laval University.

Three sites from northern Alberta were chosen for this study:

1) EMEND site (Ecosystem Management by Emulating Natural Disturbance), which is approximately 90 km northwest of Peace River (approximately N 56° 46' 13", W 118° 22' 28", GPS).

2) Slave Lake site (N 55° 17.657', W 114° 53.223').

3) ALPAC (Alberta-Pacific Forest Industries Inc.) mill site located 40 km North of Boyle (N 54° 56' 24.790", W 112° 51' 50.919", GPS).

Two sites from Québec were chosen as well:

1) St-André-de-l'Épouvante, in the Saguenay-Lac-Saint-Jean area (N 48° 15' 00.2", W 072° 00' 53.4", GPS).

2) Lac Bouchette, in the Saguenay-Lac-Saint-Jean area (N 48° 10' 20.8", W 072° 13' 50.3", GPS).

On each of the five sites, eighteen soil cores (three transects of six samples each) were originally sampled, but because of time and financial restrictions, the number of samples on each site was reduced to nine by mixing two consecutive soil cores together (samples 1 + 2, samples 3 + 4, etc.), resulting in three transects of three samples each. The number of samples used for DNA extraction was further reduced to five by random selection. The soil cores were thoroughly mixed and microcentrifuge tubes were filled with soil for further DNA extraction. Precautions were taken to prevent cross-contamination of samples.

3.2.2 DNA extraction from soil samples

Method 12 of DNA extraction described in section 2.3.3.12 of Chapter 2 was used to extract DNA from soil samples with a slight modification. About 1 g of non-dried soil from the EMEND site and Slave Lake site were used to extract DNA using 0.35 g of glass beads, 1 ml of sodium phosphate buffer, and 0.5 ml of lysis buffer (100 mM NaCl, 500 mM Tris-HCl pH 8.0, 10% SDS) buffer. Because there was no Mini-Beadbeater available, the Mixer Mill MM 300 (QIAGEN Inc., Mississauga, Ontario) with a 3 mm tungsten carbide bead was used. Some tests were done to determine the best conditions leading to the highest yield of DNA as observed on an agarose gel. Thus, samples were homogenized in the Mixer Mill three times 2 min at a frequency of 15 Hz. After each 2-min interval, the tubes were switched to the other side of the Mixer Mill for a better homogeneity because the tubes were not in a straight horizontal position. Four microlitres of RNase A was added to the homogenate and incubated for 1 h at 65°C. Tubes were then centrifuged for 3 min at 12000 rev min^{-1}. Supernatants were mixed with 0.4 vol of 7.5 M ammonium acetate (NH_4OAc), placed 10 min on ice and centrifuged 3 min at 12000 rev min^{-1}. An aliquot of 150 µl of supernatant was poured into Sephacryl S-400 HR MicroSpin™ Columns (Amersham Pharmacia Biotech Inc.) and centrifuged at 3000 rev min^{-1} for 2 min following the procedure A of General Protocol of MicroSpin™ Columns Instructions.

3.2.3 DNA extraction for PCR positive controls

DNA was extracted from *Laccaria bicolor* as described in section 2.3.5 of Chapter 2. DNA was also extracted from spores of *Glomus intraradices* following the procedure of Simon (1996a). First, the agarose medium containing spores was cut in small pieces of approximately 4 mm^2, put in a 15-ml centrifuge tube and mixed for 15 min in the presence of 100 ml of 10 mM sodium citrate. When all the agarose had melted, the tube was centrifuged for about 1 min in an International clinical centrifuge model C1 54149M-8 (USA, International Equipment CO IEC, Needham Hts, Mass). The pellet was transferred into a new 15-ml centrifuge tube and filled with sterile dH_2O and centrifuged for one min. This step was repeated once. About 100 spores were transferred in 1.5-ml microcentrifuge tubes with 50 µl dH_2O. The spores that were not immediately processed were stored at –80°C. The spores from one tube were crushed with a small plastic pestle and 50 µl of freshly made 20% Chelex 100 resin (BioRad Laboratories, Inc., USA) was added. The tube was vortexed and put in boiling water for 3 min. The tube was then transferred in liquid nitrogen for 2 min. The sample was put again in boiling water for 5 min and these steps were repeated until three freeze-thaw cycles were completed. One microlitres of DNase-free RNase A was added to the sample, which was left at room temperature for 30 min. The crude DNA extract was stored at –80°C. Because it did not remain stable for very long, the extraction had to be repeated at least every week.

3.2.4 PCR amplification

To specifically amplify DNA from endomycorrhizal fungi, a nested PCR was performed. Nested PCR improves both specificity and sensitivity of amplification and eliminates false-positive results, because in the second amplification, a set of oligonucleotide primers internal to the DNA fragment amplified in the first PCR is used. It also dilutes inhibitors present in the first PCR reaction (Berthelet *et al.*, 1996). In the first PCR (PCR#1), universal primers NS1 (5-GTAGTCATATGCTTGTCT-3) and NS8 (5-TCCGCAGGTTCACCTACGGA-3) (GIBCO-BRL®, Life Technologies) were used to amplify a portion of the eukaryotic 18S rDNA. The master reaction mix was a 25 µl

solution containing 20 mM Tris pH 8.4, 5 mM $MgCl_2$, 100 mM KCl, 2.5% Triton X-100, 0.7 U of *Taq* polymerase, 400 µM of dNTPs (Amersham Pharmacia Biotech Inc.), and 1 µM of each primer. Twenty-five microlitres of the different dilutions (1/10, 1/25, 1/100, 1/250, 1/500, and 1/1000) of the soil DNA extracts was added to the tubes. Two positive (*Laccaria bicolor* and *Glomus intraradices* DNA) and one negative (no DNA added) amplification controls were included. When the DNA Thermal Cycler 480 (Perkin-Elmer Cetus, Inc.) was used, the master reaction mix was covered with 25 µl of mineral oil, but when the PTC-225 DNA Engine Tetrad™ Cycler (Peltier Thermal Cycler, MJ Research, Inc., Watertown, Mass.) was used, no oil was added. Thin-walled reaction tubes of 0.2 ml were put in the thermocycler when the temperature reached 94°C. When using HotStarTaq™ DNA Polymerase from QIAGEN, a pre-incubation at 95°C for 15 min was included. The cycles used were the ones used by Simon *et al.* (1992) with the primer pairs NS1-NS6 (5-GCATCACAGACCTGTTATTGCCTC-3) and NS3 (5-GCAAGTCTGGTGCCAGCAGCC-3)-NS8. The first cycle was: 95°C for 2 min, 53°C for 55 s, and 72°C for 35 s. The next 13 cycles were: 96°C for 35 s, 53°C for 55 s, and 72°C for 35 s, followed by 11 cycles (96°C for 35 s, 53°C for 55 s, and 72°C for 2 min). The last 15 cycles were: 96°C for 35 s, 53°C for 55 s, and 72°C for 3 min. A final 10-min extension at 72°C was added. The DNA synthesis time for the first 14 cycles was raised to 50 s to see if it would result in an increase in PCR product yield, since the expected PCR product was longer than the one obtained with the primer pairs NS1-NS6 and NS3-NS8. PCR products from PCR#1 were separated on a 1% agarose electrophoresis gel to verify their size as described in Chapter 2.

A second PCR (PCR#2) using the specific primer VANS1 (5-GTCTAGTATAATCGTTATACAGG-3) and the universal primer NS4 (5-CTTCCGTCAATTCCTTTAAG-3) was conducted to specifically amplify a 1.10 kb region specific to rDNA from vesicular-arbuscular or endomycorrhizal fungi (Claassen *et al.*, 1996). For this, some of the dilutions of the PCR product reaction obtained using the primers NS1 and NS8, i.e. 1:24, 2:23, 4:21, 5:20, 8:17, 10:15, 15:10, 20:5, and 25:0 (µl of PCR product: µl ddH_2O), were used as templates. The intensity of the PCR product #1 was used to determine which dilutions to use, thus, not all of the dilutions were used

every time. Alternatively, the PCR product was purified from the PCR reaction using the QIAquick PCR purification Kit from QIAGEN, diluted, and used as a template. Not all the same dilutions were used every time. The PCR master mix was the same as for the PCR#1. Tubes were put in the thermocycler when the temperature reached 94°C. When using HotStarTaq™ DNA Polymerase from QIAGEN a pre-incubation at 95°C for 15 min was included. The cycles used were the ones from Claassen *et al.* (1996) with the same pair of primers except that 30 or 40 cycles were used instead of 50 cycles. This reduction in the number of cycles was done to decrease the chances of obtaining non-specific products. Temperature cycles were 94°C for 1 min, 55°C for 2 min, and 72°C for 2.5 min. A final 10-min extension at 72°C was added. In an attempt to eliminate non-specific and NS1-NS8 PCR products, the annealing temperature was raised from 55°C to 58°C or 60°C. Primers VANS1 and NS4 were also used directly on the DNA extracts.

PCR products #2 were separated on a 1% agarose electrophoresis gel to verify their size. The 1.10 kb PCR products were then purified from the gel using the QIAquick Gel Extraction Kit from QIAGEN, because there were always some non-specific products or products from PCR#1 present in the PCR reaction. The concentration of the 1.10 kb fragments was then determined in the presence of Hoechst 33258 (bis-benzimidazole) using the TKO 100-Dedicated Mini Fluorometer (Hoefer Scientific Instruments, San Francisco) according to the manufacturer's instructions.

3.2.5 Cloning of PCR products

Because the PCR#2 was specific to endomycorrhizal fungi in general, the band purified from the gel could contain DNA fragments from more than one species. A good way to separate individual PCR products is to clone them in a bacterial vector. The PCR products #2 were cloned using the QIAGEN PCR Cloning^{plus} Kit. The purified PCR product mixture was ligated with the pDrive Cloning Vector. The protocol provided with the kit recommended a PCR product concentration 5 to 10 times higher than the concentration of the pDrive Cloning Vector, but the yield of the purified PCR product obtained with the QIAquick Gel Extraction Kit allowed a maximum concentration of 1.5

times higher than the one of the cloning vector. The ligation mixture was then combined with QIAGEN® EZ Competent Cells for transformation. Each bacterial cell integrates one recombinant vector containing a single PCR product. The high copy number plasmid used as a vector in the QIAGEN PCR Cloning^{plus} Kit is replicated between 300 and 500 times in each cell. The pDrive vector contains a gene conferring resistance to ampicillin and one conferring resistance to kanamycin, as well as the LacZ α-peptide gene (compatible with the blue/white screening selection). Therefore, when plating the competent cells on Luria Bertani (LB) agar plate supplemented with ampicillin, the synthetic compound 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal), and isopropyl β-thiogalactopyranoside (IPTG), only the cells containing the vector conferring the resistance to ampicillin could grow. Moreover, only the cells transformed with the recombinant plasmid (containing the PCR product #2) were white because the insertion of the PCR product is located inside the LacZ α-peptide gene, which inhibits the production of the LacZ α-peptide responsible for the blue coloration when the N-terminally deleted β-galactosidase is expressed in the presence of X-gal and IPTG.

To prepare the X-gal stock solution (40 mg ml^{-1}), 400 mg of 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside was disolved in 10 ml diethylformamide. The solution was protected from light by wrapping it in aluminum foil and was store at -20°C. For the preparation of the IPTG stock solution (100 mM), 238.3 mg of isopropyl β-thiogalactopyranoside was dissolved in 10 ml dH_2O. The solution was filter-sterilised and stored in aliquots at -20°C. The ampicillin stock solution (30 mg ml^{-1}) was prepared by dissolving 5 g of ampicillin sodium salt in 50 ml dH_2O. The solution was filter-sterilised and stored in aliquots at 4°C. To prepare the kanamycin stock solution (30 mg ml^{-1}), 1.5 g of kanamycin monosulphate salt was dissolved in 50 ml dH_2O. The solution was filter-sterilised and stored in aliquots at 4°C.

The Luria Bertani (LB) agar plates were prepared by dissolving 10 g of tryptone, 5 g of yeast extract, 10 g of NaCl, and 15 g of agar in 950 ml dH_2O. The pH of the suspension was adjusted to 7.0 before the addition of 1000 ml of dH_2O. The suspension was then autoclaved for 20 min and allowed to cool to 55°C before 1 ml of ampicillin stock

solution, 2ml of X-gal stock solution, and 0.5 ml of IPTG stock solution were added and mixed. The LB suspension was poured into the plates, allowed set, inverted, and stored at 4°C under sterile conditions. Freshly poured plates (<1 week old) were used for plating cells because long-term storage of plates can result in decreased antibiotic activity. Twenty microlitres, 100 µl and about 150 µl were plated on petri dishes. The plates were kept at room temperature until the transformation mixture was absorbed into the agar and they were then inverted and kept at 37°C overnight. To enhance blue colour development, the petri dishes were further incubated at 4°C for a few hours.

3.2.6 Purification of the recombinant plasmids

Five clones (white colonies) from each PCR reaction, and thus from each soil sample, were selected and individually placed in 5 ml of liquid LB medium supplemented with kanamycin (only 5 clones were selected due to time and financial constrains). The liquid LB medium was prepared by dissolving 10 g of tryptone, 5 g of yeast extract, and 10 g of NaCl in 950 ml dH_2O. The pH was adjusted to 7.0 before the addition of 1000 ml of dH_2O. The solution was autoclaved for 20 min and allowed to cool to 55°C before addition of 1 ml of kanamycin stock solution.

The cultures were incubated overnight in an Innova® Model 4300 incubator shaker (New Brunswick Scientific Co., Inc.) at 37°C and 220 rev min^{-1}. Tubes were then centrifuged 16 min at 4°C and 6500 rev min^{-1} in a Sorvall® RC 5C *Plus* centrifuge (Sorvall® Instruments, Dupont). Plasmids were then purified from the bacterial culture using the QIAprep Spin Miniprep Kit from QIAGEN.

After purification, plasmids were digested with *EcoR*I restriction endonuclease enzyme and run on a 1% agarose gel to make sure that the right PCR fragment (1.10 kb) was integrated in the plasmid, because non-specific PCR products or primer-dimers could have been ligated into a plasmid as well.

3.3 Results

The the amplification with primers NS1 and NS8 on the purified DNA extract did not give consistent results when the DNA extracts were purified with method 12. No bands or bands of very weak intensity were usually obtained. The increase of the extension time from 35 s to 50 s did not improve the yield of PCR product.

Direct specific amplification of endomycorrhizal DNA from soil with primers VANS1 and NS4 did not yield any PCR product. However, a first round of PCR amplification with primers NS1 and NS8 followed by amplification with VANS1 and NS4 sometimes yielded PCR products when using the same DNA extracts (data not shown), sometimes even when no PCR product could be seen on the gel from the amplification with NS1 and NS8. When the PCR product #1 was purified from the gel and then used as a template, many non-specific products were obtained (data not shown). The increase of the annealing temperature in PCR #2 from 55°C to 58°C or 60°C did not produce any differences.

Moreover, once a PCR amplification product was obtained and purified from the gel to isolate it from other PCR reagents, such as dNTPs, non-used primers, primer-dimers, or non-specific products, its concentration was very low compared to that suggested by the manufacturer of the cloning kit (QIAGEN Inc., Mississauga, Ontario). The protocol provided with the kit recommended a PCR product concentration 5 to 10 times higher than the concentration of the pDrive Cloning Vector, but the yield of purified PCR product obtained with the QIAquick Gel Extraction Kit gave a concentration of up to 1.5 times higher than the one of the cloning vector. Nevertheless, some recombinants were obtained as seen by the lack of blue color of some of the bacterial colonies.

The enzymatic digestion of the purified plasmid showed that, in most cases, the inserted fragment was 1.10 kb, i.e. the size of the region amplified with VANS1 and NS4 (Claassen *et al.*, 1996). However, the sequencing of the PCR product, and thus, the identification of the endomycorrhizal fungi, was not done.

3.4. Discussion

The lack of consistent amplification with the primer pair NS1-NS8 could be due to the presence of contaminants that inhibit PCR, a too small concentration of the target DNA in comparison with the total DNA present, a weak pairing of the primers with the target DNA, or because of non-optimal PCR conditions, which could differ from the optimal conditions when the DNA extract is pure and not composed of a mixture of DNA from many different organisms.

It has already been noted that fungal DNA had to be first amplified with fungus-specific primers prior to group-specific or species-specific amplification. For example, Hamelin *et al.* (1996) found that amplification of species-specific PCR product directly from DNA extracted from roots was not very consistent. They have suggested that this phenomenon might be due to the variability in the ratio of target DNA to non-target DNA. Thus, a nested PCR approach might be essential to get consistent and reproducible results when amplifying DNA from endomycorrhizal fungi. The use of the universal primers NS1 and NS8 increased the population of DNA molecules compared to inhibitors and prokaryotic DNA, thereby increasing the number of 18S rRNA molecules and, thus, reducing the competition of other DNA molecules for the endomycorrhizal-specific primer pair VANS1-NS4. However, if this first round of amplification with NS1-NS8 favoured amplification of certain DNA molecules but not of DNA from endomycorrhizal fungi, this step would not be as efficient.

Another limitation that can be encountered during PCR is that more than one 18S rRNA gene sequence was found to occur in a single spore of *Scutellospora* species. More surprisingly, the sequences contained different variants of the sequence corresponding to the VANS1 primer, usually varying by three bases over its 21 bp length, indicating that the VANS1 site is not conserved (Clapp *et al.*, unpublished - as described by Sanders *et al.*, 1996). These sequence differences could reduce the efficiency of annealing the VANS1 primer to the AM fungi DNA, and could explain the problems that have previously been encountered when using this primer to amplify from field samples

(Clapp *et al.*, 1995). This also questions the validity of using VANS1 primers for the phylogenetic classification of the Glomales (Sanders *et al.*, 1995).

It has been proposed to separate bacteria from fungi prior to DNA extraction to reduce the presence of competitive DNA in the PCR reaction. Fægri *et al.* (1977) have developed a rapid fractionated centrifugation technique that resulted in a fraction containing 50-80% of the bacteria, and another fraction containing all the fungi together with soil debris and the rest of the bacteria. Bingle and Paul (1986) have described a method to separate fungal hyphae from soil, which recovers only 20% of the fungal hyphae. In both cases, only the fungal hyphae are recovered, but the technique may miss fungal species only present in the form of spores. Also, separation of fungal components prior to DNA extraction would lengthen the procedure leading to PCR products or could miss some of the fungal species present in the sample.

The problems encountered during the PCR amplification emphasize the need to assess the detection limit of the DNA extraction method, i.e. the number of DNA molecules from a particular organism necessary to be detected with the present and/or improved PCR conditions, the development of primers with enhanced specificity for glomalean fungi, and the optimization of the PCR conditions. To determine the minimum number of DNA molecules in the soil that can be detected by PCR when extracted with method 12, soil samples could be inoculated with bacterial cells containing a known amount of fungal DNA molecules. For this, a truncated fungal PCR fragment could be produced, cloned in a low copy number plasmid and inserted in a bacterial vector. Thus, a known number of bacterial cells, each cell containing a known number of plasmids, each plasmid containing one copy of the truncated fungal PCR product could be used to inoculate soil samples. The truncated fungal DNA could be differentiated from native fungal DNA by the difference in the fragment size. However, the difference in cell lysis efficiency between a bacterial cell and a fungal cell might bring some bias in the determination of the detection limit of method 12 with endomycorrhizal fungi, but it would still give a general idea. Moreover, running parallel PCR with primers specific to other organisms could help to determine if the failure to produce PCR products with the primer pair NS1-

NS8 and from endomycorrhizal fungi with VANS1-NS4 is due to the absence of the target DNA in the sample (if the primers specific to other organisms produce PCR products) or if it is due to the presence of inhibitors.

3.5. Conclusion

Despite the success of method 12 (described in section 2.3.3.12 of Chapter 2) to yield DNA of suitable purity from four soil types for PCR amplification using the primer pair ITS-F and ITS4, the PCR amplification of DNA from *P. tremuloides* forests using the primer pair NS1 and NS8 did not give consistent results. A second amplification with VANS1 and NS4 using PCR products from the amplification with NS1 and NS8 sometimes gave PCR products, but again, no consistent results were obtained. Another problem was that the purification of PCR products from agarose gel electrophoresis yielded concentrations of PCR fragments many times smaller than the concentration recommended by the manufacturer of the cloning kit. Nevertheless, some clones containing the 1.1 kb fragments corresponding to endomycorrhizal fungi PCR products were obtained. Therefore, the present procedure would require improvements in order to successfully analyse the molecular diversity of endomycorrhizal fungi (or any other microorganisms) communities in natural environments.

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CHAPTER 4

SUMMARY AND FUTURE WORK

In the present study, a method of soil DNA extraction for PCR was developed. An attempt was made to determine the diversity of endomycorrhizal fungi species in trembling aspen (*Populus tremuloides* Michx.) stands using molecular tools instead of conventional morphological methods. The methodology used here was to: I) extract DNA directly from soil samples from aspen stands, II) amplify endomycorrhizal DNA by PCR, III) clone the resulting PCR fragments of the correct size, and IV) sequence the cloned fragments for analysis.

Four types of soils with different chemical and physical properties were sampled and divided into their different horizons, for a total of nine horizons. A total of thirteen methods of direct DNA extraction were tried, some with the nine horizons, some with only a few of them. The method using Sephacryl S-400 HR MicroSpin™ columns (Amersham Pharmacia Biotech), which used relatively few chemicals and included a combination of chemical and physical lysing procedures, was found to be the most robust and rapid to yield DNA of a quality suitable for PCR.

Some physical and chemical soil characteristics were determined and related to DNA yield and quality, and PCR amplification success. The A_{260}/A_{230} ratio, an indicator of humic substance contamination, was significantly affected by the method of DNA extraction used, and was negatively correlated with the percentage of conventional humic acid content (HA-A) when using methods 1 and 4, and negatively correlated with the percentage of clay-associated humic acid (HA-B) when using method 5. The fact that the percentage of neither HA-A nor HA-B was negatively correlated with the A_{260}/A_{230} ratio when using methods 12 and 13 might be an indication that these two methods were effective in eliminating humic compounds from the DNA extracts. The A_{260}/A_{280} ratio, an indicator of protein contamination, was significantly affected by the method of DNA

extraction used but was not correlated with any of the soil characteristics determined in this study. The logarithm of the DNA yield (log[DNA yield]) was not significantly affected by the method of DNA extraction, but the fact that the percentage of HA-A was positively correlated with the log(DNA yield) when using methods 1, 4, and 5 indicated that the values calculated using the A_{260} were overestimated, probably due to the absorbency of HA-A at this wavelength. Again, when methods 12 and 13 were used to purify DNA, the percentage of HA-A was not positively correlated with the log(DNA yield), another fact that might indicate that the two methods efficiently removed humic substances from the DNA extracts. More importantly, the PCR amplification success was significantly affected by the method of DNA extraction (method 12 having the highest rate of success) and was negatively correlated with the percentage of total nitrogen in the soil. However, the percentage of total nitrogen explained only 8.85% of the variability of the PCR success. Thus, other factors not studied here might have had a higher influence on PCR amplification of DNA, such as the presence of other inhibitors of *Taq* polymerase and/or of non-target DNA.

Thus, the developed method of direct DNA extraction from soil using the Sephacryl S-400 HR MicroSpin™ columns was used to assess the species composition of endomycorrhizal fungi present in five trembling aspen stands (three in Alberta and two in Québec). After extracting the total DNA from the soil samples, the eukaryotic DNA was amplified using the universal primer pair NS1-NS8. The PCR product obtained from the first amplification was then amplified using the primer pair VANS1-NS4 specific to endomycorrhizal fungi. However, the PCR amplification did not give consistent results. Also, when purifying the PCR products prior to cloning, the concentration of pure PCR products obtained was between 3 and 5 times smaller than the optimal concentration needed, which could lead to a non optimal amount of recombinant DNA, and thus, could miss some endomycorrhizal species.

Therefore, because of the problem brought about by competitive PCR using a mixture of DNA template that might still contain some PCR inhibitors, it would be important to determine the sensitivity of the developed DNA extraction method. Also, the PCR

conditions could be improved and new primers developed to enhance the specificity of the PCR amplification for endomycorrhizal fungi. Moreover, another method of PCR product purification should be used to increase the recovery of the PCR fragments to avoid underestimation of the diversity of endomycorrhizal fungi species in trembling aspen stands. Only after incorporating these improvements can the described method of direct DNA extraction be used on soil samples to accurately estimate the endomycorrhizal fungi (or any other microorganism) diversity.

To further analyse the efficiency of method 12 for direct DNA extraction, evaluation of the cell lysis performance with the chemical and physical lysis procedures used in this study should be done. This could be accomplished by microscopic evaluation of the cells before and after cell lysis using acridine orange to stain the cells (Moré *et al.*, 1994). The lysis efficiency depends on the organism studied, because some organisms, such as small coccoid bacteria, can be left intact following SDS and bead-mill homogenization (Moré *et al.*, 1994). Also, further work should be done to evaluate the detection limit of the PCR amplification using DNA extracted with method 12, which might vary depending on the organisms and sequences targeted, the PCR conditions used, as well as the primer specificity. Also, more should be known about the accuracy of using such small samples (less than 1 g of soil) in representing microbial communities as they occur in the field. Zhou *et al.* (1996) also mentioned that it is important to recognize that no single method of cell lysis or purification will be appropriate for all soils and experimental goals.

4.1 Literature cited

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